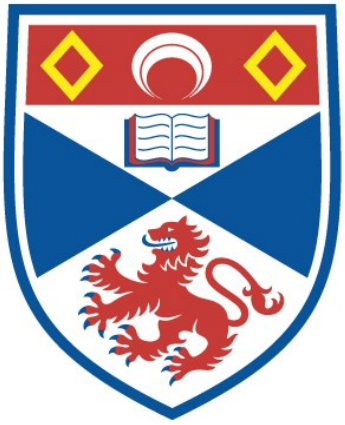
CONTRIBUTION TO THE STUDY OF THE INTERCELLULAR MATRIX OF CARTILAGE

Augusto Serafini-Fracassini

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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CONTRIBUTION TO THE STUDY OF THE INTERCELLULAR MATRIX OF CARTILAGE

by

A. SERAFINI-FRACASSINI

A thesis presented to the University of St. Andrews for the Degree of Doctor of Philosophy

Department of Biochemistry,
University of St. Andrews.

1967

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Professor G. R. Tristram.

C E R T I F I C A T E

I hereby certify that A. SERAFINI-FRACASSINI has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews) and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of Padova (Italy) in October 1953, and graduated with the degree of Doctor of Medicine, first class with medal, in November 1959.

From December 1959 to September 1965 I held the post of lecturer in the Department of Histology, Padova University.

In September 1965 I accepted the post of assistant lecturer in the Department of Biochemistry, St. Andrews University, and I was appointed to a full lectureship in the same Department as from February 1966.

ACKNOWLEDGMENTS

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Financial support from the Scottish Home and Health Department during the last year of this research is gratefully acknowledged.

I also thank Dr. J.W. Smith for his advice and collaboration in part of this work.

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Finally, I wish to thank my wife, Laura, for carrying out the amino acid analyses and, above all, for her constant understanding and encouragement.

C O N T E N T S

INTRODUCTION

The major components of the extracellular phase of bovine septal cartilage are collagen and a protein-polysaccharide complex, the macromolecular structure of which has been extensively studied since Schatton and Schubert (1954) showed that aqueous extracts of this tissue contained a high-molecular-weight component consisting of chondroitin sulphate apparently bound to a non-collagenous protein.

The protein-polysaccharide once extracted from the tissue, by high speed homogenization in large volumes of water (Malawista and Schubert, 1958), can then be separated into a light fraction (PP-L) and a heavy fraction (PP-H) by centrifugation (Gerber, Franklin and Schubert, 1960). Analytical ultracentrifugation and zone electrophoresis studies have shown that below pH 10, PP-L behaves as a single entity (Schubert, 1964). On the other hand, there is no evidence that PP-H is a single compound: on the contrary, it has been suggested recently (Schubert, 1965) that it may consist of PP-L united to collagen by ester bonds.

At a pH of 12.4, PP-L splits irreversibly into polysaccharide (CSA) and 'protein' moieties (Malawista and Schubert, 1958; Gerber et al., 1960; Muir, 1964). However, analysis of the 'protein' moiety has shown that only 68% of its nitrogen is recoverable as amino acids (Partridge and Davis, 1958), and on papain digestion some 15% of keratosulphate is liberated (Gregory and Roden, 1961).

Webber and Bayley (1956) estimated, by sedimentation and viscosity methods, that the molecular weight of calcium chloride-extracted protein-polysaccharide complex was 1×10^6. They also suggested that the complex had a random coil configuration and that it consisted of some 20 chondroitin sulphate molecules, of molecular weight approximately 4×10^4, linked by polypeptide chains in an end-to-end arrangement. Bernardi (1957 a,b), working with a similar preparation, confirmed the random coil configuration and reported a molecular weight of 1.98×10^6. Mathews and Lozaityte (1958) pointed out that light-scattering and viscosity data supported the existence of a rod-like fundamental molecular unit of molecular weight 4×10^6. They proposed a comb-like model in which the protein moiety was visualized as a core, 3700 Å

in length, along which 62 chondroitin sulphate chains were uniformly distributed. This hypothesis of a continuous protein core was fundamentally confirmed by a study of the kinetics of degradation by hyaluronidase and papain of a preparation of protein-polysaccharide (Cessi and Bernardi, 1965). However, Partridge, Davis and Adair (1961) have suggested that PP-L, in fact, consists of smaller units having a molecular weight of about 7.5×10^5, and that in each such unit a protein core, with a molecular weight of 120,000, gives attachment to some twenty-three CSA chains, each with a molecular weight of 28,000.

It is evident, therefore, that various preparations of chondromucoprotein exhibit widely varying molecular weights (Webber and Bayley, 1956; Bernardi, 1957 a,b; Mathews and Lozaityte, 1958; Partridge et al., 1961; Luscombe and Phelps, 1967). Mathews and Lozaityte (1958) pointed out that chondroitin sulphate-protein molecules may form aggregates of molecular weight up to 5×10^7 by lateral and end-to-end association in which additional non-collagenous protein probably participates. On the

other hand, Meyer (1966) suggested that the crude complexes of molecular weight above 1 x 10^6 and high protein content could be made up of smaller complexes bridged by basic proteins. This hypothesis was supported by the observation that chondromucoprotein preparations purified by chromatography on DEAE-cellulose have been reported to have a protein content of approximately 7.6% (Partridge, Whiting and Davis, 1965) and a molecular weight as low as 2.4 x 10^5 (Partridge, 1966). Similar results were obtained by Buddecke, Krötz and Lanka (1963), who, by purification with cetylpyridinium chloride, obtained a material of molecular weight 5.5 x 10^5, but characterized by a protein content of approximately 16%. The last-named authors also showed that, in the presence of Cu^{2+}, Ca^{2+} or Co^{2+}, molecular aggregates of molecular weight up to several million formed.

Finally, Mashburn, Hoffman, Anderson and Meyer (1965) reported that even the mildest methods of extraction yield complex of sufficient electrophoretic inhomogeneity to prevent the definition of a discrete protein-polysaccharide complex. Similarly, Rosenberg, Johnson and Schubert (1965) showed that chondromucoprotein

PP-L from human costal cartilage can be separated into three fractions by the use of La^{3+}. Loewi (1964) and Loewi and Muir (1965) indicated that porcine chondromucoprotein could be separated by electrophoresis into immunologically distinct constituents.

The collagenous nature of the fibres in mature mammalian cartilage does not seem to be in doubt, since they have been shown to have the same amino acid composition as collagen from other sources (Dixon and Perkins, 1956). Nevertheless, the fibres in intact cartilage, other than articular cartilage, do not exhibit the characteristic repeating band pattern of collagen after uranyl acetate or phosphotungstic acid staining (Fitton Jackson, 1964).

Because protein-polysaccharide has never been visualized in intact cartilage, its relationship to the collagen fibres of that tissue is unknown. Most authors make the tacit assumption that there is no linkage between the two. On the other hand, the

suggestion by Schubert (1965) and Partridge and Davis (1958) that part, at least, of the protein-polysaccharide is bound to collagen is supported by several facts, namely the difficulty of extracting readily soluble protein-polysaccharide from whole cartilage, the peculiar staining reaction of the collagen in that tissue, and the chemical characteristics of PP-II.

Binbinder and Schubert (1951), in their study on the binding of dyes and mucopolysaccharides by insoluble collagen *in vitro*, reported that the amount of chondroitin sulphate fixed by collagen varies as a function of the pH, at which the reaction is carried out, with a maximum at pH 3.5. They interpreted these results by suggesting an electrostatic interaction taking place between the sulphate groups only of the polysaccharide and the cationic groups of collagen. And because they were unable to detect any binding above pH 7 they concluded there was no basis for believing that salt-like compounds could form, at neutral pH, by interaction between mucopolysaccharides and collagen *in vivo*.

The role of electrostatic forces on the formation and stabilization of the mucopolysaccharide-collagen complex has been emphasized by Mathews (1965) in his investigation, by free solution electrophoresis at pH 7, of the reversible association of acid mucopolysaccharides and solubilized collagen. This collagen preparation had a near-native structure with a molecular weight ranging between 1 and 20 million. The failure of heparin and chondroitin sulphate (Mw 18,000) to form complexes with collagen at I = 0.4, led the author to the conclusion that chain length as well as number and kind of interacting charged groups per molecule are very important features. On the other hand, since the complex formation was destroyed by prior heating of solubilized collagen, the author suggested that high molecular weight or internal structure of the fibrous protein are probable requirements.

This thesis reports:

i) the electron microscopic examination of PP-L, obtained from an aqueous extract of bovine nasal cartilage, by using a new "staining" procedure. This work is concerned mainly with the assessment of the length of the protein-polysaccharide macromolecule and of the number of chondroitin sulphate chains linked to the protein core;

ii) the electron microscopic study of the relationship of protein-polysaccharide macromolecules to collagen in PP-N;

iii) the physicochemical characterization, chemical composition and amino end-group analysis of the protein-polysaccharide macromolecules examined by electron microscopy in PP-L;

iv) the distribution of collagen and protein-polysaccharide in both nasal and articular cartilage;

v) the investigation, <u>in vitro</u>, of the reaction of purified tendon collagen with protein-polysaccharide complex, chondroitin sulphate, heparin and high-molecular weight dextran sulphate.

MATERIALS AND METHODS

Extraction of chondroitin sulphate-protein complex

Fresh bovine nasal septa were cleaned, planed into stripe and extracted by the procedure of Malawista and Schubert (1958). Chondromucoprotein was fractionated by high-speed centrifugation into PP-L and PP-H by the method of Gerber et al. (1960), all manipulations being carried out as quickly as possible at 4°C to avoid proteolytic degradation. PP-L was precipitated from its solution in 0.15M.KCl by addition of 2 volumes of ethanol. This material is referred to, in the present paper, as crude PP-L (PP-L-C).

The CSA and protein fractions of PP-L-C were prepared by the procedure of Malawista and Schubert (1958).

Electron microscopy of PP-L-C and PP-H

In the course of the present study, it was established that bismuth nitrate was a satisfactory electron microscope "stain" for the carbohydrate moiety of protein-polysaccharide and its derivatives. The dimensions of these carbohydrate moieties are below the practical limits of resolution in the electron microscope.

But because they are all polyelectrolytes of considerable flexibility, it is to be expected that after exchange of a sufficient number of their monovalent counterions by Bi^{3+}, they would assume a coiled configuration in which they would be visible in the electron microscope as dark particles. The method thus permitted a study of the morphology of PP-L-C and its derivatives within the limits discussed above, and also an investigation of the relationship of protein-polysaccharide to collagen in both PP-H and whole cartilage.

Two preparations of bismuth nitrate were used. In the first, 1 g. bismuth nitrate was dissolved in 10 ml. 2M nitric acid and this was made up to 200 ml. with distilled water, giving an 0.5% solution in 0.1M nitric acid at pH 1.2. This will be referred to as "aqueous bismuth nitrate". In the second preparation 1 g. bismuth nitrate dissolved in 10 ml. 2M nitric acid was made up to 200 ml. with acetone. This will be referred to as "bismuth nitrate in acetone".

Protein-polysaccharide and its derivatives were dissolved in distilled water in various concentrations, and these solutions were then mixed with an excess of

one or other of the two bismuth preparations. The precipitates which formed were spun down and washed several times with water or acetone. After redispersion, the suspensions were sprayed onto carbon-coated grids which were then dried in a desiccator. Most of the grids were examined without further staining, but some of the PP-H grids were additionally stained with 2% phosphotungstic acid.

Purification of chondroitin sulphate-protein complex

For the preparation of the bismuth-purified PP-L (PP-L-Bi), the protein-polysaccharide complex was precipitated from a 2% (w/v) solution of PP-L-C in 0.5M. KCl by slow addition of 4 volumes of $Bi(NO_3)_3$ in acetone (1 g. of $Bi(NO_3)_3$ was dissolved in 10 ml. of 2N.HNO_3 and this was made up to 200 ml. with acetone). The flocculent white precipitate was collected by centrifugation and washed with acetone and with distilled water. The precipitate was then stirred with 0.25M.KCl for three hours at 4°C. Insoluble inorganic material was discarded after centrifugation and 2 volumes of ethanol was added to the supernatant, which was kept overnight at 4°C, when a fine precipitate formed. This precipitate was collected by centrifugation,

dissolved in 0.6M.KCl, clarified by centrifugation and re-precipitated at 4°C by the addition of ethanol. Finally, PP-L-Bi was dissolved in 0.15M.KCl and centrifuged at 78,000 $g_{av.}$ for 30 minutes. The clear supernatant was exhaustively dialysed against distilled water at 4°C and freeze-dried. The average yield was 80% of the starting material.

Precipitation of PP-L-C by cetylpyridinium chloride on a cellulose column followed by fractional elution of the complex with a salt gradient was adopted as an alternative purification procedure. The product was cetylpyridinium chloride-purified PP-L (PP-L-CPC). The method used was that of Scott (1960), as modified by Antonopoulos, Borelius, Gardell, Hammström and Scott (1961). A linear gradient of $MgCl_2$ in 0.05% (w/v) cetylpyridinium chloride was used for the elution of the column. The eluate was monitored for its E_{260} by a base-compensating automatic recorder, and for its polyanion content by the turbidimetric method of Scott (1960). PP-L-CPC, eluted as a symmetrical peak, was separated from cetylpyridinium chloride by cooling and filtration (Scott, 1960). The purified protein-polysaccharide complex was precipitated by ethanol, dialysed against distilled water and then

freeze-dried. The average yield was 75% of the starting material.

Assay of proteolytic activity of protein-polysaccharide preparations.

The proteolytic activity of various PP-L preparations was tested according to the method described by Anson (1938), by using as substrate bovine haemoglobin that had previously been exhaustively dialysed against distilled water and freeze-dried. Citrate-phosphate buffers were used over the range pH 2 - 8.

Chemical determinations

Ash and moisture were estimated as described by Bastoe and Courts (1963).

Total nitrogen was estimated by the method of Chibnall, Rees and Williams (1943).

For hexosamines, hydrolysis was carried out by heating the sample in a sealed tube under N_2 with 4N.HCl (2 ml. of acid/mg. of material) at 105°C for 8 hours. After hydrolysis, excess of acid was removed in a rotary film evaporator at 50°C.

Total hexosamine was estimated by the method of

Cessi and Piliego (1960). Controls were run to allow for losses occurring during both hydrolysis and removal of acid.

Ogston (1964) pointed out that estimation of hexosamine in complex polysaccharides may give low results because other substances present may be converted, during hydrolysis, into products that subsequently decrease the yield of chromogen in the reaction with acetylacetone. To check this possible source of error, samples of bovine haemoglobin, casein gelatin and serum albumin were hydrolysed under the conditions specified for hexosamine estimation. The recovery of known amounts of galactosamine added to these hydrolysates was then determined. The results showed that haemoglobin did not interfere even in 1000-fold excess. Gelatin lowered the yield if present in greater amount than 200-fold excess, but albumin, in the range 200-2000-fold excess, had the opposite effect. None of the proteins tested caused any significant interference in the protein concentration range of PP-L.

The two hexosamines were separated by the method of Partridge and Elsden (1961).

Thiol group was estimated by the method of Ellman (1959).

Amino acid analysis

Solutions of various PP-L preparations, in 200 times their own weight of constant-boiling HCl, were heated in sealed tubes under N_2 at 110°C for 24 hours. Additional hydrolyses were carried out for 36 and 72 hours with sample 1 and sample 5 (Table 1). Excess of acid was removed from the hydrolysates in a rotary film evaporator, the temperature of the water bath being maintained at 30°C.

Alkaline hydrolysis for tryptophan was carried out by the procedure of Brenner, Niederwieser and Pataki (1965).

Amino acid analyses were carried out with a Technicon Auto-Analyser. Since the samples contained large amounts of hexosamines, it was found necessary to change the buffer gradient to avoid galactosamine merging with valine. This was done by lowering the pH of the first buffer from 2.875 to 2.750 and by adding to the first two chambers of the Autograd 5 ml. and 3 ml. respectively of methanol.

Amino end-group analysis

Samples of approximately 200 mg. of dried protein-polysaccharide were dissolved in 50 ml. of 0.15M.KCl in the reaction vessel of a Radiometer pH-stat set at pH 8.4. The microsyringe was filled with 0.25N.NaOH and N_2 was bubbled through the solution. After the pH had been stabilized, a 5 ml. volume of a freshly prepared 5% (v/v) solution of 1-fluoro-2,4-dinitrobenzene in ethanol was added. The reaction was followed on the recorder and stopped when the rate of titration had levelled off. The viscous product was then exhaustively dialysed against distilled water at 4°C. The dinitrophenylated material (DNP-PP-L) was collected by freeze-drying as it was soluble even on acidification of the medium and was not precipitated by the addition of 10 volumes of acetone. Acid hydrolysis of DNP-PP-L for 12 hours at 110°C in a sealed tube with a 200-fold excess of constant-boiling HCl gave rise to marked humin formation. Attempts to separate the DNP-amino acids from the various artefacts by chromatography on a silicic acid column (Stoven and Tristram, 1962) were only partially

successful and losses occurred. Resin hydrolysis was therefore adopted. The procedure was essentially that described by Steven (1962). Samples (200 mg.) of DNP-PP-L were mixed with 30 ml. of a thick aqueous slurry of Dowex (X8; H^+ form; 200-400 mesh) in sealed tubes and heated for 30 hours at 100°C in an oven fitted with a device for rotating the tubes about their mid-point at 10 rev./min. α-DNP-amino acids were recovered from the resin by elution with boiling water. Amino acids and the remaining DNP-derivatives were then eluted with O.8N.NH_3 solution.

Samples of the ether-soluble DNP-amino acids were subjected to two-dimensional thin-layer chromatography according to Brenner et al. (1965) on 20 cm. x 20 cm. glass plates. The plates were developed with toluene-pyridine-2-chloroethanol-O.8N.NH_3 solution (10:3:6:6, by volume) in the first dimension and chloroform-benzyl alcohol-acetic acid (70:30:3, by volume) in the second dimension. Samples of the water-soluble DNP-amino acids and the remaining amino acids were separated by thin-layer chromatography with butan-1-ol-acetic acid-water (4:1:1, by volume) in the first dimension and phenol-water (3:1, w/v) in the second. The plates.

after development, were sprayed with 0.3% ninhydrin in butan-1-ol acidified with acetic acid. Both the resin hydrolysis products of DNP-PP-L were rehydrolysed with constant-boiling HCl for 18 hours at 105°C, in sealed tubes, and rechromatographed. This procedure was followed because, with resin hydrolysis, breakdown of protein to its constituent amino acids may not be complete and the presence of peptides could lead to misleading interpretation of chromatograms.

After development, the spots of the ether-soluble DNP-amino acids were removed according to the vacuum technique of Ritter and Meyer (1962). The DNP-amino acids were eluted from the 'thimbles' with chloroform-acetic acid (99:1, v/v) and E_{360} measured. Quantitative thin-layer chromatograms were run in quadruplicate and samples of standard DNP-amino acids were also subjected to hydrolysis and chromatography to allow correction for losses.

Analysis of the acetone supernatant after precipitation of PP-L-Bi

The acetone supernatant was examined for free amino acids and peptides after PP-L-Bi had been collected by

centrifugation. Acetone was removed in a rotary film evaporator; the residue was then neutralized with NaOH and extracted twice with butan-1-ol previously equilibrated with N-HCl. The extracts were evaporated to dryness and then desalted on a column of Dowex 50 (X8; H^+ form; 200-400 mesh) (Smith, 1960). The eluate from the column was evaporated to dryness, dissolved in a small volume of 80% (v/v) acetone and subjected to thin-layer chromatography with the solvent systems described for the analysis of the water-soluble DNP derivatives. The developed plates were sprayed with both acidified ninhydrin in butan-1-ol and Morgan-Elson reagent (Waldi, 1965).

Physicochemical measurements

The various PP-L preparations were dissolved in, and dialysed against, 0.15M-KCl before analyses.

A Spinco model E analytical ultracentrifuge with a Schlieren optical system was used for ultracentrifugation. The constant-temperature control was set at 20°C.

Partial specific volume was calculated from density measurements performed at 20°C with two 10 ml. pycnometers by the procedure of Washburn and Smith (1934).

Viscosity measurements were made at 20°C with a capillary viscometer constructed as described by Schachman (1957).

Nasal and articular cartilage

Analytical methods

Samples of bovine nasal and articular cartilage were lyophilized and dried to constant weight *in vacuo* over concentrated sulphuric acid. The residual moisture content (which was always less than 3%) and the sulphated ash content were estimated by the methods of Eastoe and Courts (1963).

Acid hydrolysis was found to cause excessive destruction of uronic acid and hexosamines, and consequently the desiccated cartilage was hydrolysed by the resin hydrolysis procedure of Anastassiadis and Common (1958). Maximum yields of uronic acid were obtained after 2 hours, whereas maximum yields of hexosamines and hydroxyproline occurred after 30 hours. The eluates from the resin were analysed for hexosamines by the method of Cessi and Piliego (1960), for uronic acid by the carbazole technique of Downess (1967) and for hydroxyproline by the procedure

described by Serafini-Cessi and Cessi (1964). Control hydroxyproline determinations on samples of cartilage which had been hydrolysed for 24 hours at 110°C in sealed tubes with redistilled 5.7N hydrochloric acid were in close agreement with the results obtained after resin hydrolysis. No elastin was found in either cartilage by the procedure of Neuman and Logan (1950).

Histological methods

Small pieces of articular cartilage were obtained from the metatarsophalangeal joints of 18-month-old bullocks within a few minutes of death. Some of this material and thin fragments of nasal cartilage were fixed immediately in 1% osmium tetroxide in veronal/acetate buffer at pH 7.2, and after embedding in Araldite, sections were stained with 2% aqueous uranyl acetate or 1% aqueous phosphotungstic acid. Other pieces were stained without previous fixation by bismuth nitrate. They were immersed in 0.1M nitric acid for 2 hours and then in 0.5% bismuth nitrate in 0.1M nitric acid for a further 24 hours. The material was then fixed in neutral formalin and embedded in Araldite. The sections were examined without further staining.

Interaction of insoluble collagen and sulphated macromolecules

Preparation of collagen

Ox Achilles tendon was cut into pieces which were washed first with water and then with 1M.NaCl. The fibres were normally dissociated and extracted with 1M-NaCl for 3 days at +1°C. They were then washed with successive changes of distilled water for 48 hours, and extracted three times with ethanol:chloroform (1:3, by vol.). The dry material was ground to a fine powder in a mill before use.

The purified collagen contained ash 0.03, moisture 12%.

Physicochemical characterisation of heparin

The heparin used was obtained from Wilson Ltd. (Lot 136651). The molecular weight was determined in a Spinco model E analytical ultracentrifuge, using the Ehrenberg adaptation of the Archibald approach to equilibrium method. Heparin was dialyzed for 12 hours in 1/15OM-phosphate buffer pH 7, to which sodium chloride was added to give an ionic strength of 0.2.

The experiments were performed at 29,500 r.p.m. on samples containing approximately 1.5 x 10^{-3}g./ml. heparin. Partial specific volume was calculated from measurements made in a density gradient column prepared according to the method of Linderstrøm-Lang (Hvidt, Johansen, Linderstrøm-Lang and Vaslow, 1954; Linderstrøm-Lang and Lanz, 1935-8; Linderstrøm-Lang, Jacobsen and Johansen, 1938-41) as modified by Miller and McGurren Gasek (1960).

Refractive increments were measured in a differential refractometer (Polymer Consultants Ltd.) at a wavelength of 546 mμ at 20°. Calculations were made on a dry weight basis.

Viscosity studies were made at 20° with a capillary viscometer constructed as described by Schachman (1957). The measurements were made over a concentration range of 0.8 - 1.9 x 10^{-2}g./ml.

Physicochemical characterisation of chondroitin sulphate

The chondroitin sulphate used was obtained from Sigma Chemicals Ltd. (Lot 15B-0010).

Chondroitin sulphate was dialyzed for 12 hours in 0.15M-phosphate buffer pH 7, with 0.2M.NaCl. Viscosity

studies were made as described above.

Physicochemical characterisation of dextran sulphate

The dextran sulphate used was obtained from Sigma Chemicals Ltd. (Lot 15B-2320; type 500S). Molecular weight was determined in a Hewlett-Packard/Mechrolab Membrane Osmometer. The constant-temperature control was set at 20°. The membranes used were produced by Schleicher and Schuell Co. (Type B19).

Dextran sulphate was dissolved in, and dialyzed against:

1) 0.1M-phosphate buffer, pH 6.5;

2) 0.1M-phosphate buffer + 0.4M.NaCl, pH 6.5.

The measurements were made over a concentration range of 4 to 18 x 10^{-3} g./ml.

Estimation of sulphate

The method used for sulphate estimation was that described by Giellman and Tölg (1960). The principle employed is the reduction of sulphate to hydrogen sulphide which is distilled over, in a hydrogen atmosphere, into 2N.NaOH giving rise to sodium sulphide. Sodium sulphide is then titrated with cadmium solution, using dithizone as indicator.

Binding of acid polysaccharides by collagen

In order to measure the degree of binding of chondroitin sulphate, heparin, PP-L-Bi and dextran sulphate by collagen, the following procedure was used.

Weighed samples of 125 mg. of dry collagen were shaken for three hours at 20° with 13.5 ml. of polysaccharide solutions in 0.01M-phosphate-citrate buffer. The pH range examined was from 3 to 6.6. The solutions were then centrifuged. The amount of polysaccharide bound was calculated from the unbound polysaccharide in the supernatant as measured from sulphate estimations carried out before and after shaking.

The shift in pH due to reaction was also recorded.

Electron microscopy

The various residues were stained with bismuth nitrate in acetone before electron microscopic examination.

R E S U L T S

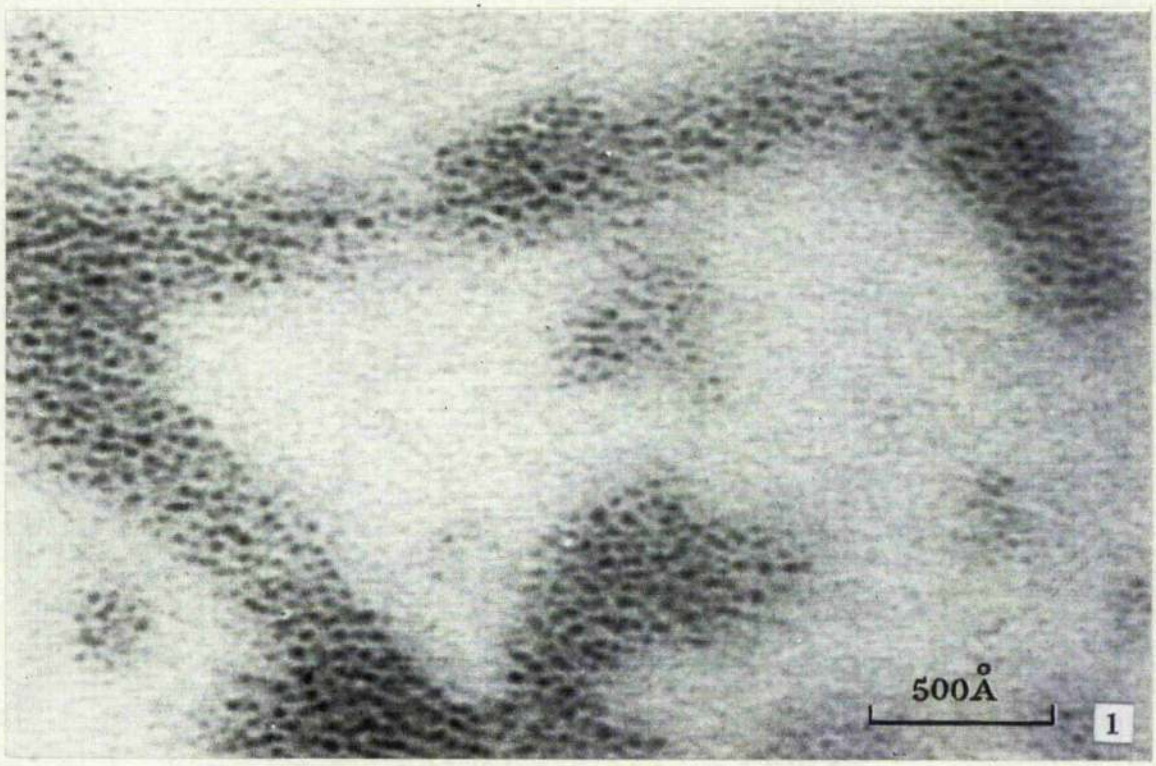
Electron microscopy of PP-L-C and its derivatives

(a) Chondroitin sulphate (CSA)

After precipitation of an 0.5% solution of CSA by aqueous bismuth nitrate, the morphology of the precipitate is as shown in figure 1. The material consists of discrete groups of dark spheroidal particles which have an average diameter of about 23Å, and there is no evidence of isolated individual particles between the groups.

The pH of the mixture of CSA and bismuth nitrate was 1.4. Gurd and Murray (1954) have demonstrated that at such a pH little if any ion binding is to be expected from ionizable carboxyl groups. Consequently, it is considered probable that the bismuth ions are entirely bound to the sulphate groups of CSA. The nitrogen content of the bismuth salt of CSA was determined by the method of Ma and Zuazaga (1942) as 2.38%, whereas that of the CSA disaccharide unit ($C_{14}O_{14}H_{19}NS$) is 3.06%. From these figures it can be

Figure 1



The CSA fraction of PP-L, precipitated with aqueous bismuth nitrate and sprayed on grid.

(x 433,000)

calculated that the bismuth salt contains 78% CSA and 22% bismuth, and that the ratio of the number of CSA disaccharide units to the number of bismuth ions is 1.6. It is probable therefore that many of the bismuth ions are bound to more than one sulphate group on the same or on different CSA chains. And this, in turn, suggests that the discrete groups of particles in Figure 1 represent networks of CSA chains joined to one another by intermolecular cross-links. This concept is in conformity with the work of Katchalsky and Zwick (1955) who, in their study of the effects of divalent cations on polymethacrylic acid gels showed that many of the bound cations were involved in the cross-linking of functional groups located on one or more polymethacrylic acid chains.

It is considered, therefore, that the individual 23 Å dark particles in Figure 1 indicate those segments of the cross-linked CSA chains which have assumed a coiled configuration as a result of the neutralization of their net charge. Consequently, although Figure 1 demonstrates that bismuth ions are bound to CSA, probably to the sulphate groups, it is considered that the size of the stained particles gives no direct

indication of the size or form of the individual CSA molecules.

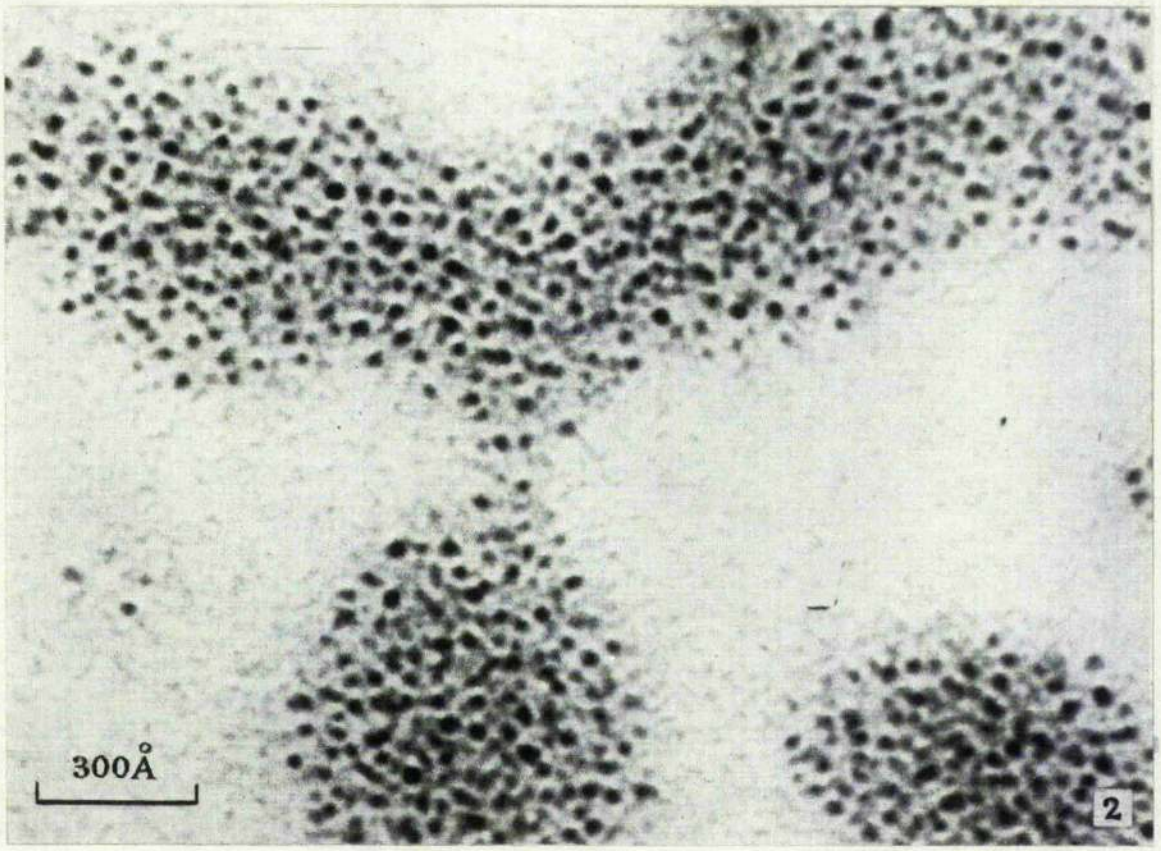
(b) "Protein" fraction

When an 0.5% solution of the "protein" fraction is precipitated by aqueous bismuth nitrate and the suspended precipitate is sprayed onto grids, the material takes the form shown in Figure 2. The appearance is essentially similar to that of CSA in Figure 1. The spheroidal dark particles have a similar average diameter of about 23 Å, and are arranged in discrete groups.

Albersheim and Killias (1963), in their study of cellular staining reactions with bismuth nitrate, found that the cation was not bound to protein to any significant extent, and in the present investigation (see below) no staining of collagen has been observed. It is probable, therefore, that the staining of the "protein" fraction in Figure 2 is due to the binding of bismuth to the sulphate groups of the keratosulphate moiety rather than to its protein part.

It seems likely that the reaction of the bismuth ions with keratosulphate is essentially similar to that postulated for bismuth and CSA, though the

Figure 2



The "protein" fraction of PP-L, precipitated with aqueous bismuth nitrate and sprayed on grid.

(x 630,000)

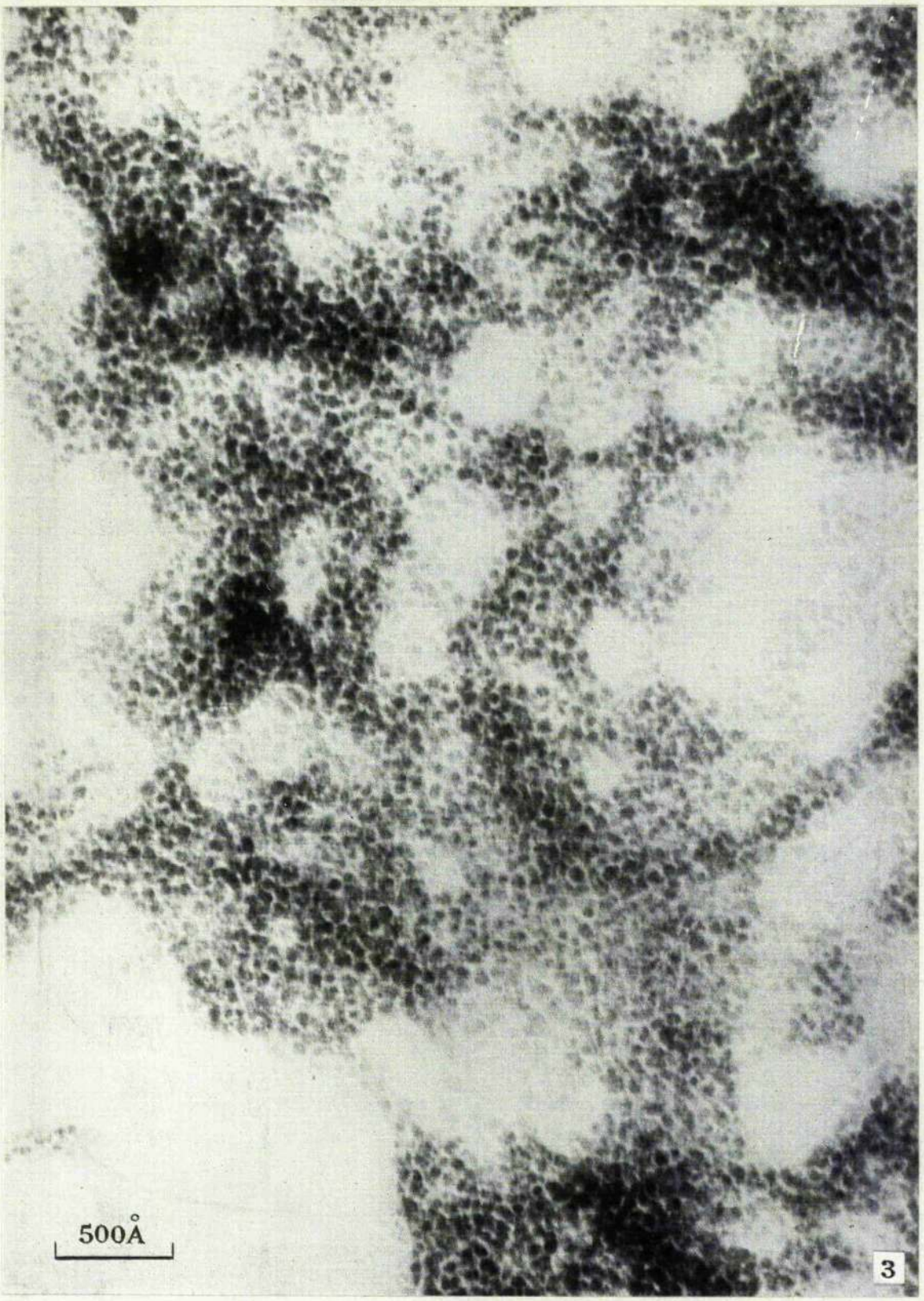
comparative rigidity of the protein moiety of the "protein" fraction probably reduces the extent of intermolecular cross-linking. It is considered therefore that the visible particles in Figure 2 represent coiled segments of keratosulphate chains, carried by networks of cross-linked protein molecules, and consequently that the size of these particles gives no indication of the size or form of the keratosulphate molecule.

(c) The light fraction of proteinpolysaccharide(PP-L-C)

(1) The morphology of PP-L, after precipitation of an 0.5% solution by aqueous bismuth nitrate, is shown in Figure 3. It resembles the comparable preparations of CSA and of the "protein" fraction (Figures 1,2) in that it consists of groups of closely packed, dark, spheroidal particles. However, the average size of the particles is 47 $\mathring{A}$, 50% being between 45 and 50 $\mathring{A}$, so that their average volume of 55,000 $\mathring{A}^3$ is about ten times that of the CSA or "protein" particles.

It is considered that each group of particles represents a network of interweaving PP-L macromolecules,

Figure 3



PP-L precipitated with aqueous bismuth nitrate and sprayed on grid (x 350,000)

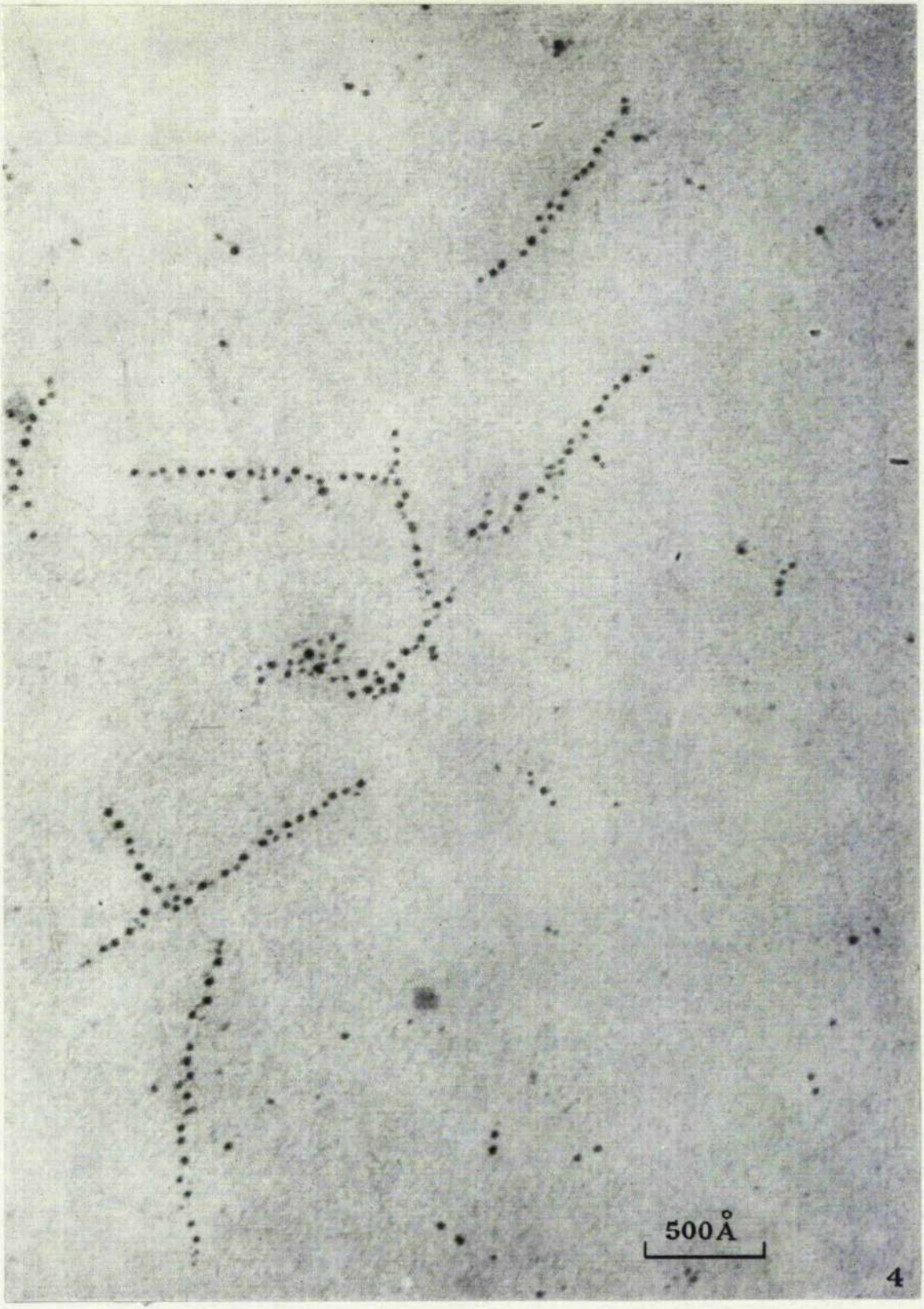
cross-linked by bismuth through their carbohydrate moieties, and that each individual dark particle represents a carbohydrate segment of this network in coiled configuration. Although the preparations of CSA and PP-L cannot be directly compared, a comparison of the "protein" fraction and PP-L may be valid, since both are visualized as interweaving protein cores, cross-linked through their carbohydrate moieties. It may be, therefore, that the greater average size of the PP-L particle compared with the "protein" particle is only an expression of the greater proportion of carbohydrate in the former material. Whatever the explanation, it is probable that the average size of the PP-L particle has no direct relationship to the molecular weight of either PP-L or its component parts.

(2) The morphology of individual PP-L macromolecules, which is obscured by the use of aqueous bismuth nitrate, is made evident when precipitation is produced by bismuth nitrate in acetone; 5 ml. of a 1% solution of PP-L in distilled water was added to 90 ml. of bismuth nitrate in acetone giving a final acetone concentration of 90%. The resulting

precipitate was separated by centrifugation, washed repeatedly in 90% acetone and sprayed onto grids. Its appearance is shown in Figure 4. The great majority of the stained elements are dark particles arranged in single rows. The rows vary in length from 1100 to 1500 Å and each contains from twenty to twenty-five particles. The particles vary in diameter in the range 15 to 47 Å (average 30 Å), whereas the unstained intervals between the particles vary in length from 17 to 60 Å (average 36 Å). Moreover, there is no apparent correlation between the lengths of the intervals and the size of their limiting particles.

It seems very improbable that this arrangement of particles is fortuitous. It is considered, therefore, that the rows of particles in Figure 4 represent protein-polysaccharide macromolecules in which the protein core is unstained, and in which the dark particles represent the carobhydrate moieties in coiled configuration. Indeed, the lengths of the rows and the number of particles in each row agree very closely with the chemical findings of Partridge et al. (1961) and with the model which these authors

Figure 4



PP-L precipitated with bismuth nitrate in acetone and sprayed on grid (x 350,000)

proposed for molecular units of protein-polysaccharide having a molecular weight of 7.5×10^6.

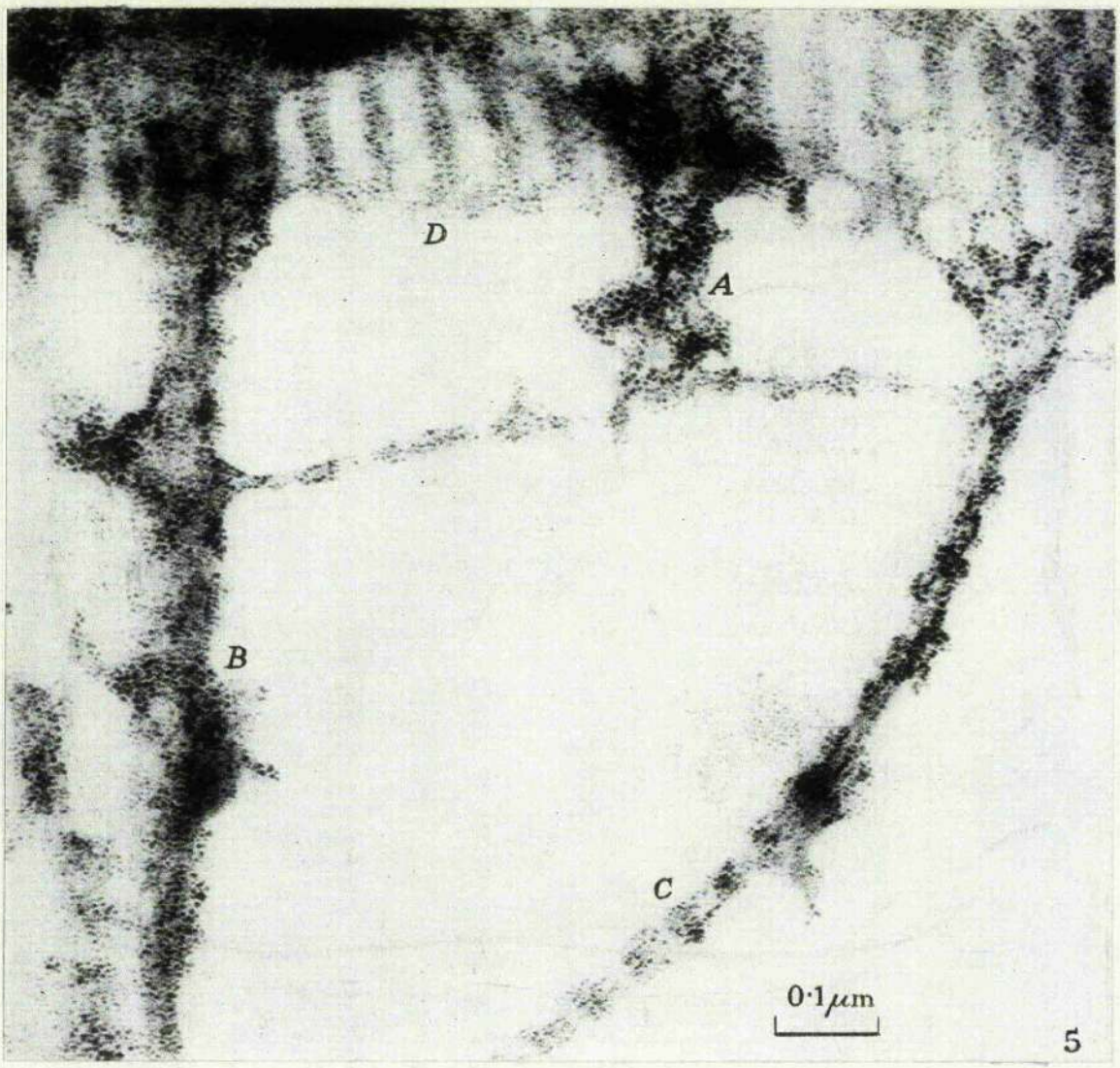
It is evident that precipitation of PP-L in acetone results in a more or less complete separation of the macromolecules. It is considered that the prevention of intermolecular cross-linking between PP-L macromolecules is a result of the greatly increased repulsion between like charges which is to be expected in a medium of low dielectric constant. But if this factor prevents intermolecular cross-linking it must also very largely inhibit cross-linking between adjacent CSA chains along the length of the protein-polysaccharide macromolecule. This concept is strongly supported both by the almost completely extended form of the macromolecules in Figure 4, and by the coincidence of the number of particles in each macromolecule and the number of CSA chains as estimated by Partridge et al. (1961). It follows, that in this preparation, each particle very probably represents an individual carbohydrate unit, the greater part of which is CSA. The fact that the particles are not of uniform size does not necessarily indicate that the CSA chains are of different lengths. Each visible

particle does not represent a coiled CSA chain in its entirety, but only that part of the coil in which the density of bismuth is above a critical level. In other words, the size of each visible particle is a function not only of the length of the corresponding CSA chain but of the final shape which the chain has assumed on the grid. One factor which influences this final shape is the dehydrating effect of acetone which makes the coiled CSA chains more compact than they would be in an aqueous medium.

Electron microscopy of the heavy fraction of protein-polysaccharide (PP-H)

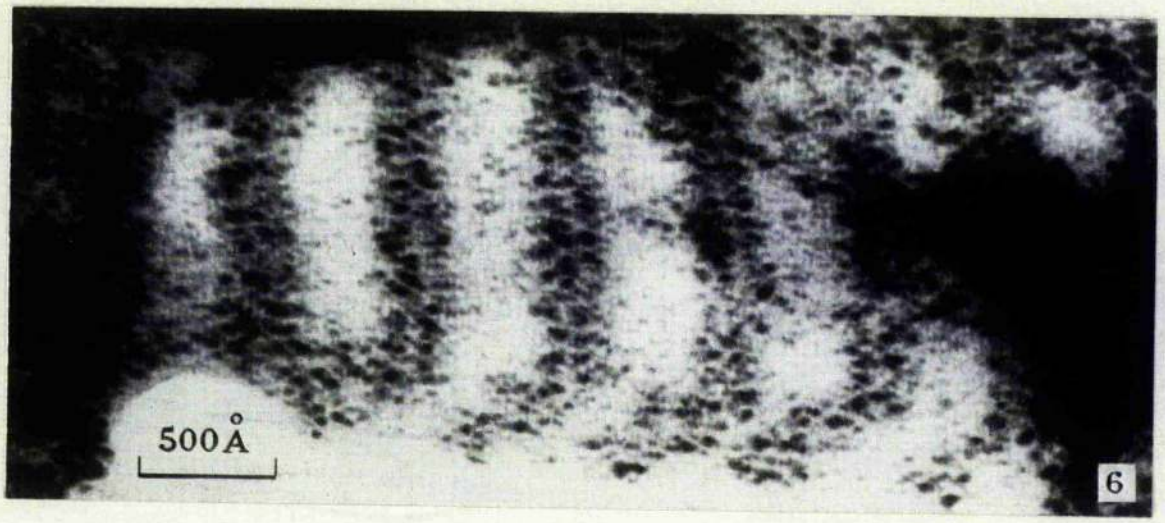
The appearance of a suspension of PP-H, treated with aqueous bismuth nitrate is shown in Figures 5 and 6. The material consists, on the one hand, of particulate areas (A in Figure 5) in which the particles appear identical to those in Figure 3, and, on the other hand, of fibres which vary in diameter from 200 to 2000 Å and are of indeterminate length. Dark particles, similar to those of the PP-L fraction, are attached to

Figure 5



PP-H precipitated with aqueous bismuth nitrate and sprayed on grid. A indicates a zone of protein-polysaccharide particles. B, C and D indicate three forms of association between protein-polysaccharide particles and collagen fibres (x 125,000)

Figure 6



Enlargement of D in Figure 5

(x 320,000)

the fibres at regular intervals of about 630 Å, and the collagenous nature of the fibres which this period indicates, is confirmed by their staining pattern with phosphotungstic acid (Figures 8 and 9).

When a suspension of purified tendon collagen was treated with aqueous bismuth nitrate, washed and sprayed onto grids, no staining of the fibres was observed. It seems evident therefore that in PP-H protein-polysaccharide macromolecules are bound to collagen in a rather specific manner. This conclusion is entirely in keeping with the composition of PP-H suggested by Schubert (1965).

The exact arrangement of the protein-polysaccharide macromolecules on the collagen fibres of PP-H varies. In some situations (B in Figure 5) dark particles are distributed along the whole length of the fibre, but are preferentially aggregated at intervals which conform to the native collagen period. Other fibres (C in Figure 5) exhibit short particle-free zones 150 to 200 Å wide, between particulate zones which are centred 630 Å apart. And on other fibres again (D in Figure 5; Figure 6) the dark particles are restricted to two narrow strips, which, with the

intervening light strip, comprise a zone 250 Å wide in each collagen period. The latter arrangement is consistent with macromolecules of protein-polysaccharide, in the form shown in Figure 4, being coiled transversely round the surface of the collagen fibre at two specific sites.

It has been noted that when protein-polysaccharide in solution is precipitated by aqueous bismuth nitrate the individual macromolecules are joined into a network by cross-linking. But it is evident that the degree of such cross-linking must be considerably influenced by any existing attachment of the macromolecules to a rigid substrate such as collagen. It is considered therefore that the different arrangements of the particles at B, C and D in Figure 5 are the consequence of different degrees of attachment of protein-polysaccharide to collagen. At D the protein-polysaccharide macromolecules are visualized as being firmly attached to collagen so that cross-linking between the macromolecules is minimal and they exhibit the same individual form as is shown by protein-polysaccharide in acetone (Figure 4). On the other hand, it is considered that at B and C in Figure 5,

the attachment to collagen is much weaker, so that, because of cross-linking between the protein-polysaccharide macromolecules, the arrangement of particles is more closely comparable to that exhibited by protein-polysaccharide in water (Figure 3). This weaker attachment may be the result of a recombination of protein-polysaccharide and collagen, separated from one another in the original extraction process.

To define the sites of attachment of protein-polysaccharide macromolecules within the native collagen period, PP-II treated with aqueous bismuth nitrate was additionally stained with phosphotungstic acid (Figures 8 and 9), and compared with purified tendon collagen stained with PTA alone (Figure 7). Using the conventional notation of Schmitt and Gross (1948) the fibre in Figure 7 exhibits the composite a band and the b_1, b_2, c_2 and d bands while the c bands and the c_1 band are just visible. The fibre shown in Figure 8 appears to be comparable to that shown at D in Figure 5, and exhibits two stripe of dark particles overlying the a and b_1 bands respectively. The upper two fibres in Figure 9 are considered to be

Figure 7

Purified tendon collagen fibre. Stained PTA and sprayed on grid. Letters indicate bands in collagen period (x 250,000)

Figure 8

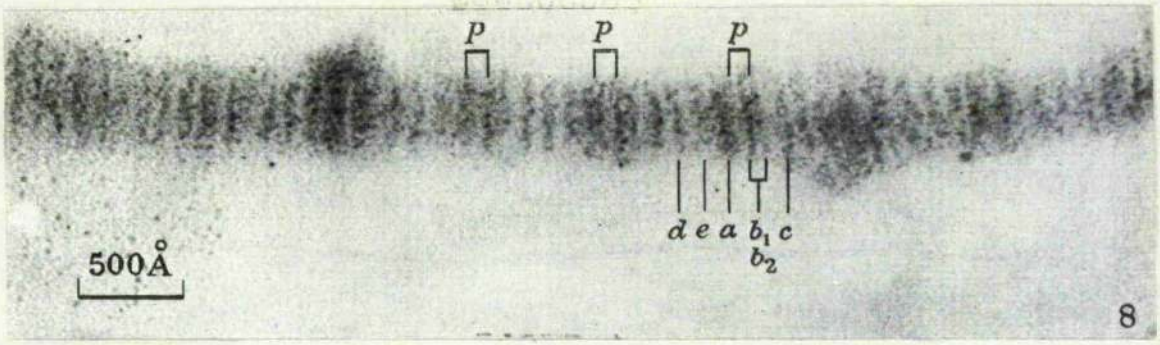


Figure 9

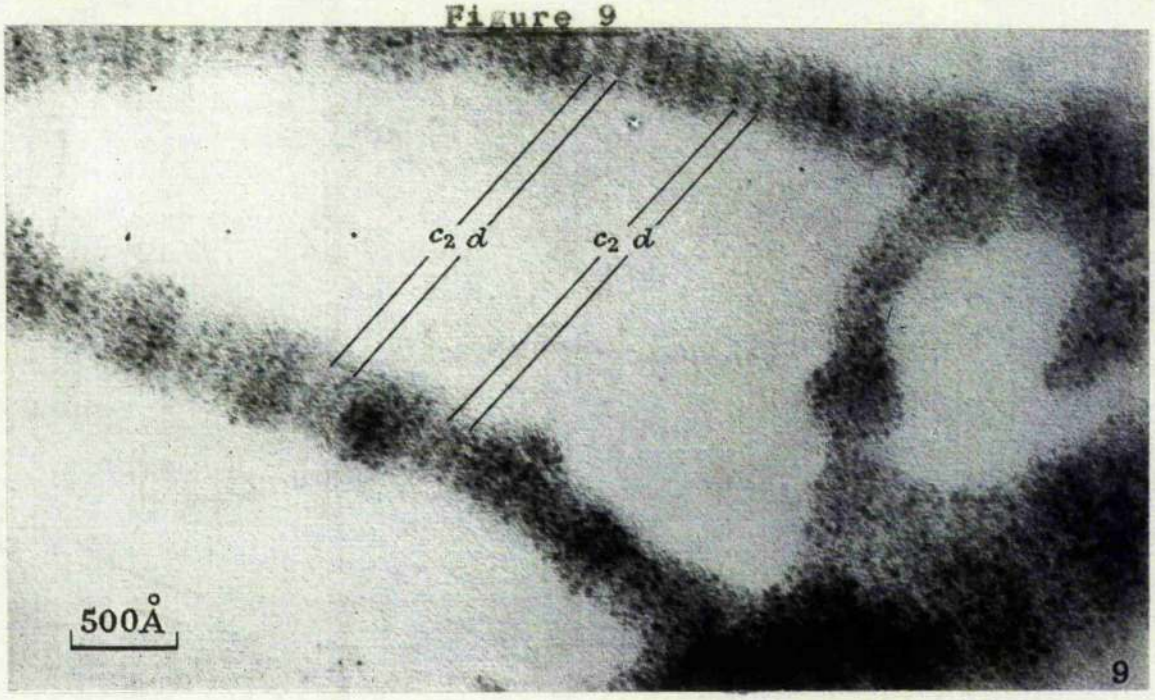


Fig. 8 PP-H. Stained PTA and subsequently treated with aqueous bismuth nitrate. Sprayed on grid. Lower letters indicate bands in collagen period. p indicates double row of protein-polysaccharide particles overlaying the a and b_1 bands.

(x 250,000)

Fig. 9 As in Figure 8. Letters indicate bands in collagen period.

(x 250,000)

comparable to that at C in Figure 5: they exhibit particulate zones covering the a and b bands and particle-free zones in which the c_2 and d bands are clearly evident.

Enzymic activity of PP-L preparations

Contamination of PP-L by proteolytic enzymes was assayed over a wide range of pH values. With PP-L-C two optima were found, at pH 2.5 and 7.5, with enzymic activities (expressed as m-equiv. of tyrosine liberated/hr./g. of PP-L) 9.0×10^{-4} and 6.0×10^{-4} respectively.

No proteolysis was detectable with PP-L-CPC and PP-L-Bi.

Protein content and composition of PP-L-C, PP-L-CPC and PP-L-Bi

To determine the protein content of the various PP-L preparations and the individual amino acid recoveries after acid hydrolysis, one sample of PP-L-C (sample 1) and one of PP-L-Bi (sample 5) were analysed after hydrolysis for 24, 36 and 72 hours at 110°C.

The compositions of the three PP-L-Bi (sample 5) hydrolysates are reported in Table 1, column (A), where the results are expressed as μg. of anhydro-amino acids/10 mg. of dry and ash-free material. A corrected protein content of 14.17% was derived by extrapolation to zero hydrolysis time of the summated amino acid contents. The same calculation procedure was applied to the analytical results of sample 1, and a corrected protein content of 20.12% was obtained. Since the extrapolation curves of sample 1 and sample 5 had identical slopes and in both cases the percentage of zero-time recovery after 24 hours hydrolysis was 90.2, a correction coefficient of 1.108 was used for all other samples examined in the calculation of their corrected protein contents from the amino acid compositions of their 24 hour hydrolysates.

The amino acid composition values of the three PP-L-Bi hydrolysates converted into g. of anhydro-amino acids/100 g. of the protein component are reported in Table 1, column (B). These values were used to estimate the influence of hydrolysis conditions on the individual amino acids. Glutamic acid, glycine,

TABLE 1

Amino acid analysis of PP-L-Bi (Sample 5)

(A) Wt. of anhydro-amino acid (μg./10 mg. of PP-L-Bi, ash- and moisture-free); (B) wt. of anhydro-amino acid (g./100 g. of the protein component); (C) recovery after 24 hr. hydrolysis (%).

Time of hydrolysis (hr.)	(A)			(B)			Corrected values	(C)
Amino acid	24	36	72	24	36	72		
Hydroxyproline	0·00	0·00	0·00	0·00	0·00	0·00		
Aspartic acid	121·59	116·50	99·79	8·58	8·22	7·04	9·36*	91·7
Threonine	74·81	71·47	55·70	5·30	5·04	3·93	6·08*	87·2
Serine	87·32	73·00	69·28	6·16	5·15	4·89	7·88†	78·3
Glutamic acid	190·19	201·38	166·57	13·42	14·21	11·75	14·21‡	94·4
Proline	116·95	107·22	88·22	8·25	7·57	6·23	9·16*	90·1
Glycine	77·88	78·38	57·87	5·50	5·53	4·08	5·53‡	99·3
Alanine	61·16	61·32	46·21	4·32	4·33	3·26	4·33‡	99·8
Valine	83·87	84·26	80·91	5·92	5·95	5·71	5·95‡	99·5
Cystine (half)	0·00	0·00	0·00	0·00	0·00	0·00	0·00	(68·8)§
Methionine	0·00	0·00	0·00	0·00	0·00	0·00	0·00	(45·4)§
Isoleucine	63·80	60·59	59·83	4·50	4·28	4·22	4·52*	99·5
Leucine	129·69	126·40	101·07	9·15	8·92	7·13	9·27†	98·7
Tyrosine	47·55	33·09	15·06	3·36	2·34	1·06	4·24*	79·3
Phenylalanine	63·45	70·85	54·17	4·48	5·00	3·82	5·00‡	89·4
Hydroxylysine	0·00	0·00	0·00	0·00	0·00	0·00	0·00	
Lysine	57·48	57·09	43·46	4·06	4·03	3·07	4·15†	97·8
Histidine	31·36	31·17	24·55	2·21	2·20	1·73	2·25†	98·2
Arginine	71·96	70·38	67·33	5·08	4·97	4·75	5·18*	98·0
Tryptophan	0·00	0·00	0·00	0·00	0·00	0·00	0·00	
Totals	1279·06	1243·10	1030·02	90·29	87·74	72·67	97·11	

Protein content extrapolated to zero time: 14·17%

* Extrapolation to zero time (24 hr., 36 hr. and 72 hr. hydrolysates).
† Extrapolation to zero time (24 hr. and 36 hr. hydrolysates).
‡ Greatest value.
§ Calculated from amino acid analyses of sample 1.

alanine, valine and phenylalanine were difficult to liberate, giving maximal values after 36 hours hydrolysis. Hydrolytic destruction of all other amino acids was apparent. Corrected concentrations of all these amino acids except four were determined by extrapolating to zero-time their concentrations in all three hydrolysates, assuming a first-order reaction over the full range of hydrolysis as suggested by the linearity of their losses. Since a non-linear destruction of serine, leucine, lysine and histidine was found, corrected concentrations of these four amino acids were calculated by extrapolation, too, but by using the 24 hour and 36 hour hydrolysates only. For each amino acid, the percentage of zero-time recovery after 24 hour hydrolysis was calculated and reported in Table 1, column (C). Again, good agreement was found with the recoveries calculated from the analytical data of sample 1.

The extrapolated protein contents and the corrected amino acid compositions of the samples that have been analysed are reported in Table 2.

Traces of tryptophan have been found, after alkaline hydrolysis, only in sample 1 (PP-L-C).

TABLE 2

Analyses of PP-L-C, PP-L-CPC and PP-L-Bi

Values are calculated as percentages of dry ash-free samples.

	PP-L-C			PP-L-CPC	PP-L-Bi		
Sample	1	2	3	4	5	1	5+1
Protein	20·12	18·18	17·48	15·96	14·17	14·14	
Nitrogen	—	5·8	—	5·8	5·6	—	
Total hexosamine (as free base)	—	29·3	—	30·0	30·5	—	
Galactosamine: glucosamine ratio	—	9·6	—	10·6	10·5	—	

Amino acid composition of the protein component

Amino acid	Wt. of anhydro-amino acid (g./100 g. of protein)						Amino acid residues (moles/1000 moles)
Hydroxyproline	0·00	0·00	0·00	0·00	0·00	0·00	
Aspartic acid	9·98	10·34	10·19	10·01	9·36	9·71	89·96
Threonine	5·46	5·45	5·58	5·56	6·08	5·55	62·45
Serine	7·11	7·31	6·81	7·46	7·88	7·87	98·19
Glutamic acid	14·59	15·21	15·23	15·93	14·21	14·29	119·83
Proline	9·32	7·82	9·11	9·14	9·16	9·23	102·80
Glycine	6·90	5·51	5·82	5·61	5·53	6·10	110·63
Alanine	5·00	5·37	4·64	4·99	4·33	4·36	66·37
Valine	5·93	6·88	6·06	5·74	5·95	5·90	64·89
Cystine (half)	1·22	Trace	Trace	0·00	0·00	0·00	
Methionine	1·54	0·00	0·00	0·00	0·00	0·00	
Isoleucine	3·95	4·87	4·92	4·50	4·52	4·80	44·70
Leucine	8·35	9·54	9·98	9·05	9·27	9·43	89·71
Tyrosine	4·16	4·38	4·70	4·20	4·24	3·67	26·32
Phenylalanine	4·43	4·94	6·36	5·61	5·00	4·98	36·81
Hydroxylysine	0·00	0·00	0·00	0·00	0·00	0·00	
Lysine	4·02	4·94	5·43	4·31	4·15	3·82	33·76
Histidine	2·19	2·11	2·26	2·22	2·25	2·22	17·69
Arginine	7·12	5·59	6·86	6·21	5·18	4·93	35·14
Tryptophan	Trace	—	—	0·00	0·00	0·00	

TABLE 3

End-group analysis of PP-L-Bi

Values are expressed as moles of amino acid/10^6 g. of protein–polysaccharide.

Aspartic acid	0·157
Threonine	0·112
Serine	0·183
Glycine	0·055
Valine	0·490
Leucine	0·592
Total	1·589

Cysteine was estimated separately, but no significant amounts were found: less than 10^{-10} mole of SH groups/mg. of PP-L.

The values for nitrogen and hexosamines contents are reported in Table 2.

Amino end-group analysis of PP-L-Bi and PP-L-C

The results of the quantitative analysis of the N-terminal amino acids of PP-L-Bi are reported in Table 3 as moles/10^6g. of protein-polysaccharide. Acid rehydrolysis confirmed that the DNP derivatives had all been released by the resin hydrolysis.

No chromatographic evidence was obtained, either before or after the second hydrolysis, for the presence of O-DNP-tyrosine.

When samples of PP-L-C were examined, a considerable increase in the number of α-DNP-amino acids was found and the total amount of these was approximately twice that of the α-DNP-amino acids of PP-L-Bi. The crude preparation was found to contain in addition to the DNP derivatives reported in Table 3 the following: DNP-glutamic acid, DNP-alanine, DNP-phenylalanine and DNP-isoleucine.

Analysis of the acetone supernatant

Chromatographic analyses carried out on the organic material liberated from PP-L-C by acetone at low pH revealed the presence of peptides, hexosamine and the following free amino acids: isoleucine, phenylalanine, tyrosine, arginine, valine, alanine, glycine, glutamic acid, leucine and aspartic acid.

Sedimentation coefficients of PP-L-CPC and PP-L-Bi

The sedimentation coefficients at infinite dilution, $S^{o}_{20,w}$, of PP-L-CPC and PP-L-Bi, obtained by linear extrapolation of 1/S or $S(\eta/\eta_o)$ against c to c = 0 by the method of least squares, were 12.41S and 12.34S respectively. The determination of $\bar{v}_{20}$ gave an average value of 0.686 ml./g. for both preparations.

Electron microscopy of bovine nasal and articular cartilages

The proportions of collagen and protein-polysaccharide vary considerably in different types of cartilage, as shown in Table 4.

In both materials the contents of uronic acid and hexosamines are approximately equal, and this indicates that nearly all the acid mucopolysaccharide of both tissues is chondroitin sulphate. The total water and sulphated ash contents are very similar in the two tissues and it is evident, therefore, that articular cartilage contains over twice as much collagen and about one-third the amount of chondroitin sulphate as compared with nasal cartilage. The collagen/CSA ratio is 5.2 in articular cartilage and 0.83 in nasal cartilage.

a) Sections of intact bovine nasal cartilage

After staining with aqueous bismuth nitrate, nasal cartilage is seen to consist of isolated and variously orientated collagen fibres, and a comparatively extensive interfibrillar matrix.

TABLE 4

Compositions of bovine articular and nasal cartilages

	Articular cartilage	Nasal cartilage
Moisture (% of fresh tissue)	78·7	74·0
Sulphated ash (% of fresh tissue)	7·7	13·1
Hydroxyproline*	9·7	4·6
Collagen*	72·4	34·5
Hexosamines*	4·7	14·8
Uronic acid*	5·0	14·9
Chondroitin sulphate*†	14·0	41·7

* Expressed as % of ash- and moisture-free material.
† Calculated from uronic acid content.

Naturally, it is comparatively seldom that a collagen fibre is found which is exactly parallel to the plane of a section, but whenever this orientation is closely approached, the appearance is as shown in Figure 10. A fibre extends throughout the length of this figure, but because it is, itself, unstained, its outline is very largely defined by the presence of transversely attached rows of dark particles. The rows are about 530 Å apart which conforms to the period which is customarily exhibited by collagen in embedded and sectioned material (Jakus, 1956). Moreover, some of the rows are double, the two parts being separated by a distinct clear zone. Thus although the definition of the particles in sections is naturally below that in sprayed material, the morphology in Figure 10 is almost exactly comparable to that already noted in PP-H at D in Figure 5 and in Figure 6. On the basis of this close morphological similarity, it is considered that in intact nasal cartilage, protein-polysaccharide macromolecules, of the form indicated in Figure 4, have a transverse surface attachment to each collagen fibre in the vicinities of the a and b_1 bands of each collagen period.

Figure 10

Figure 11

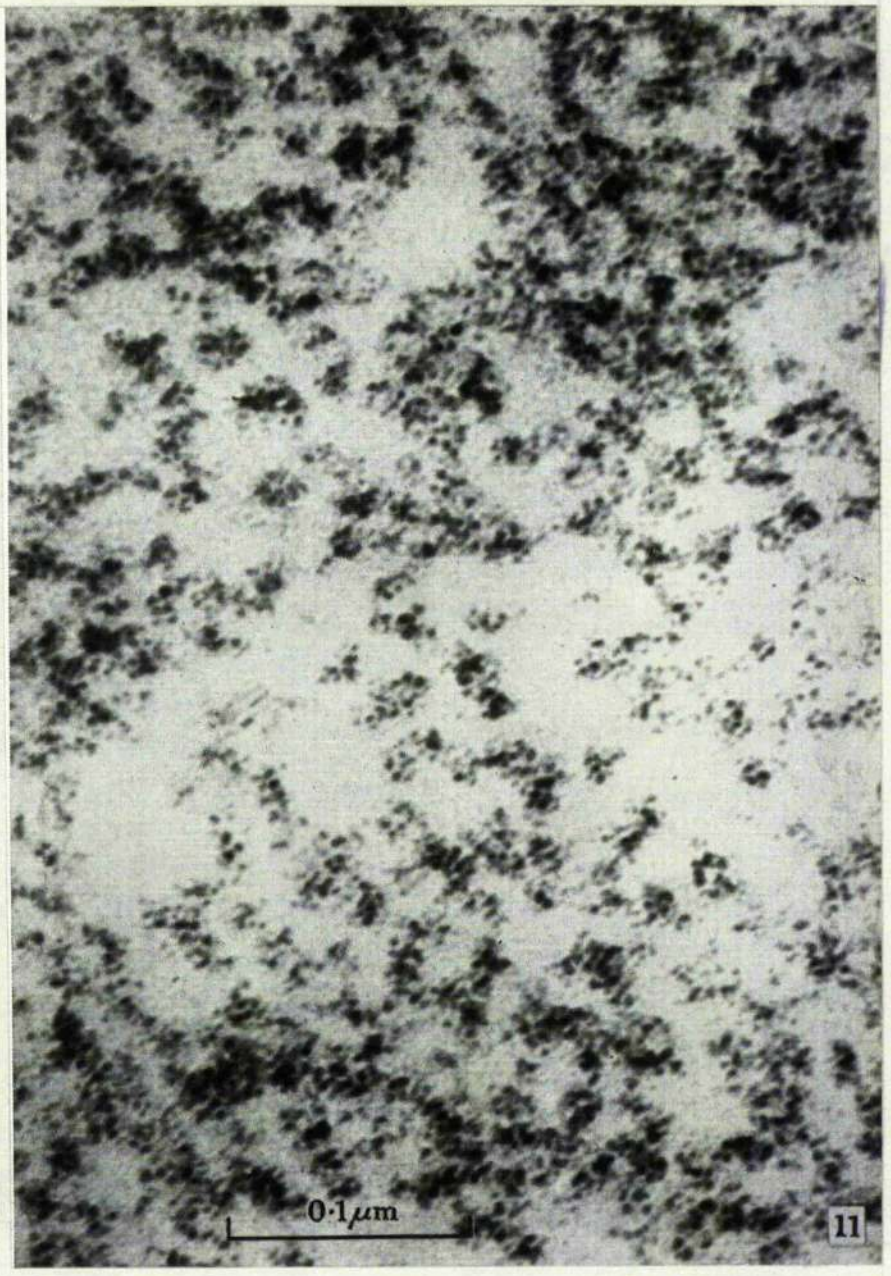


Fig. 10 Section of bovine nasal cartilage. Stained aqueous bismuth nitrate. Formalin fixation. Arrows indicate pair of protein-polysaccharide macromolecules on collagen fibre.

(x 125,000)

Fig. 11 The interfibrillar matrix of bovine nasal cartilage.

(x 290,000)

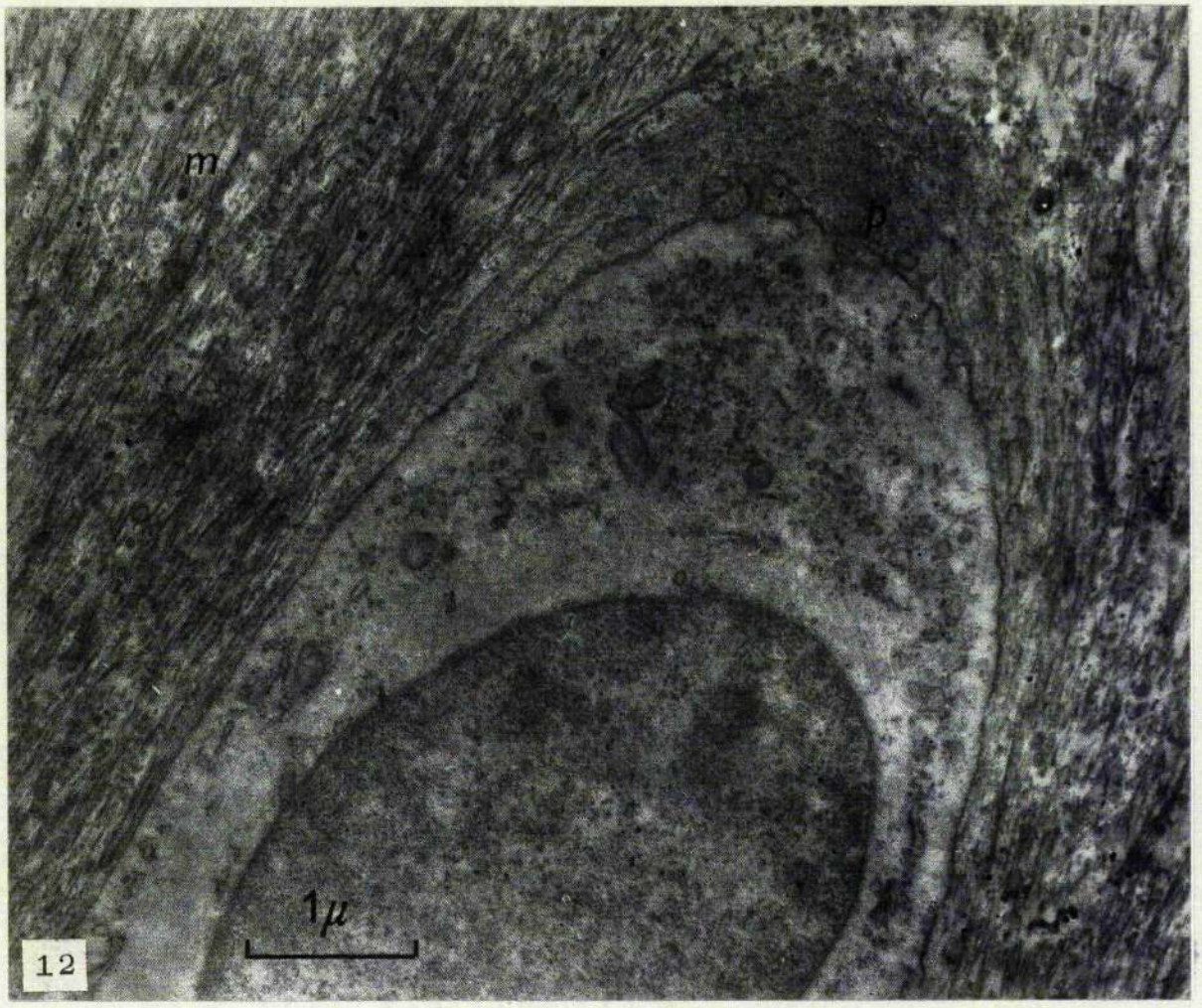
Sections through the extensive interfibrillar regions of nasal cartilage are occupied by groups of dark particles, the average dimensions of which are similar to those of the particles already noted in PP-L and PP-H. In some situations the groups are quite irregular, but in many others the particles are arranged in short single rows of four to seven. It is considered that this arrangement indicates that the interfibrillar regions are occupied by free protein-polysaccharide macromolecules. Many of the macromolecules are cross-linked by bismuth and consequently the particles are aggregated into irregular groups which, though smaller, are otherwise comparable to those in Figure 3. Other macromolecules have remained separate, as in Figure 4, probably because cross-linking with neighbouring macromolecules was inhibited by the semisolid environment in which they lie. And the small parts of these long, individual macromolecules which lie within any one section are visible as the short rows of particles already noted.

b) Sections of intact bovine articular cartilage. Ultrastructure after osmium fixation

The general appearance of bovine articular cartilage

after osmium fixation and uranyl acetate staining is shown in Figures 12 - 14. The chondrocytes exhibit the features which have been described in detail by Godman and Porter (1960), Silberberg, Silberberg, Vogel and Wettstein (1961), Davies, Barnett, Cochrane and Palfrey (1962), Sheldon and Kimball (1962), Revel and Hay (1963), Silberberg, Silberberg and Feir (1964), and Palfrey and Davies (1966). In particular, the processes of the cells are characteristically blunt and smooth and are quite distinct from the sharp spicule-like projections which are associated with contraction of the cell from the surrounding matrix as a result of poor fixation. The main part of the extracellular matrix (m) exhibits numerous straight fibres, between which the interfibrillar spaces are clear. Between the cells and the main matrix, and sharply demarcated from both, is a narrow region of distinct appearance (p) which will be designated the pericellular zone. This varies considerably in size on different aspects of the ovoid cells, being wide at the poles (Figure 12) and in the intervals between closely adjacent cells (Figure 13), but hardly appreciable around the equator (Figure 14).

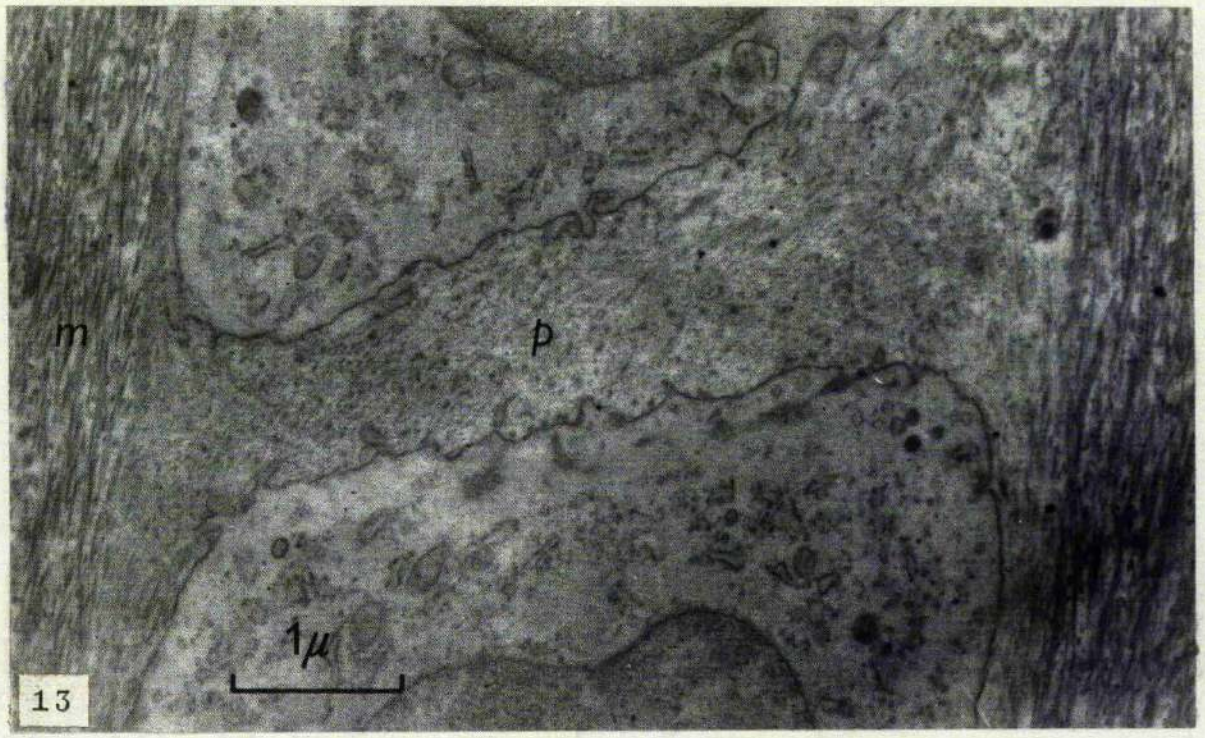
Figure 12



Bovine articular cartilage. Osmium fixation, stained uranyl acetate.

(x 20,000)

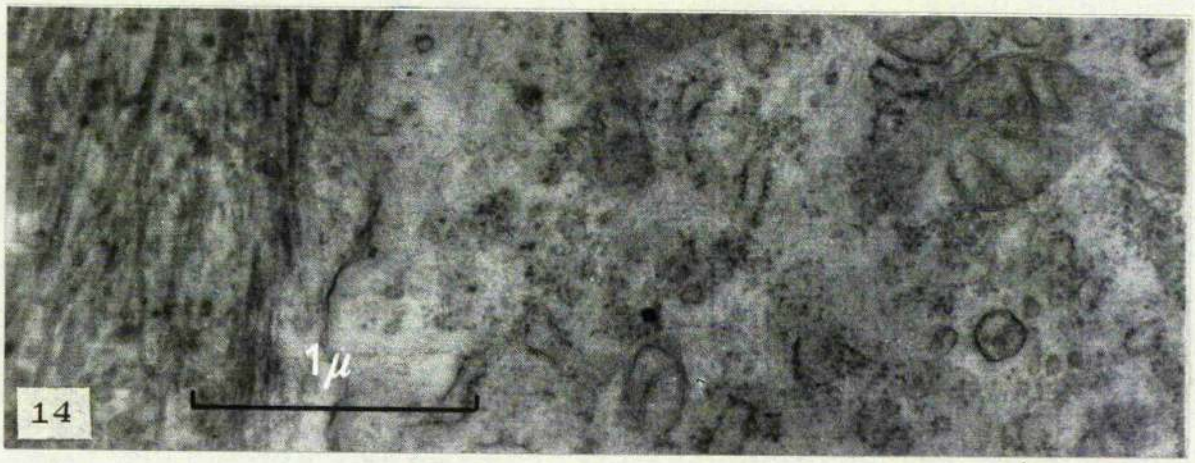
Figure 13



Bovine articular cartilage. Osmium fixation, stained uranyl acetate.

(x 20,000)

Figure 14



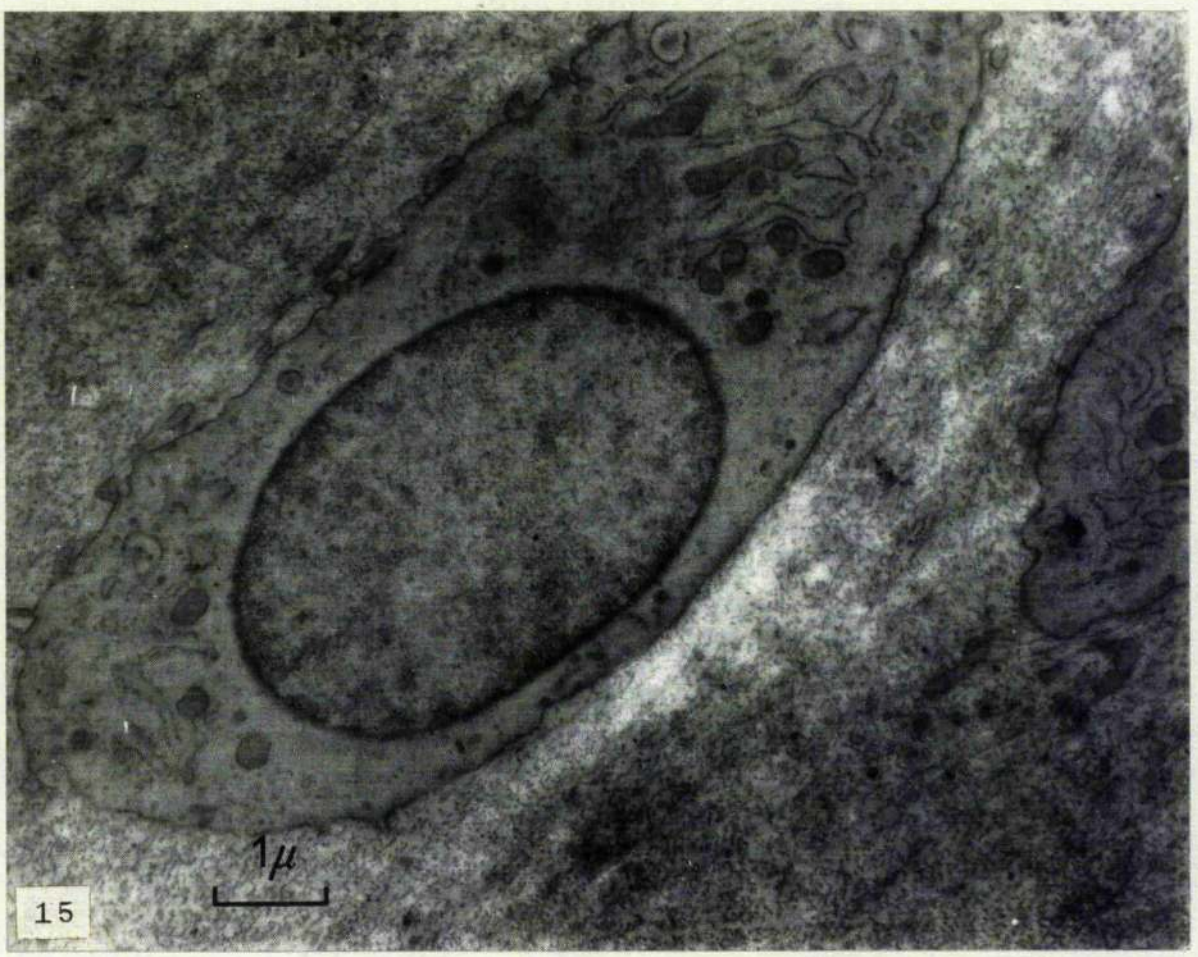
Bovine articular cartilage. Matrix adjacent to equator of cell. Osmium fixation, stained uranyl acetate.

(x 34,000)

At a higher magnification and after PTA staining (Figures 16, 17) it is apparent that the fibres of the main matrix exhibit some degree of preferential orientation. The fibre diameter varies evenly in the range 250-900 Å, but the average fibre diameter is constant throughout the region, right up to the edge of the pericellular zone. Some of the fibres are uniformly grey in appearance whereas others exhibit the banded pattern typical of native collagen. The relative numbers of fibres showing these two appearances suggest that typical collagen staining may be restricted to those fibres which are actually traversed by the surfaces of the section. There is no evidence of any interfibrillar material.

At this higher magnification the pericellular zone (Figure 17) contains a mass of fine fibres with diameters ranging from 100 to 250 Å. The fibres follow irregular rather than straight courses and give no suggestion of any preferential orientation. Although they do not exhibit the staining pattern of native collagen, it seems evident, from their form and the known composition of cartilage matrix, that the fibres are, in fact, collagenous in nature. Their exact

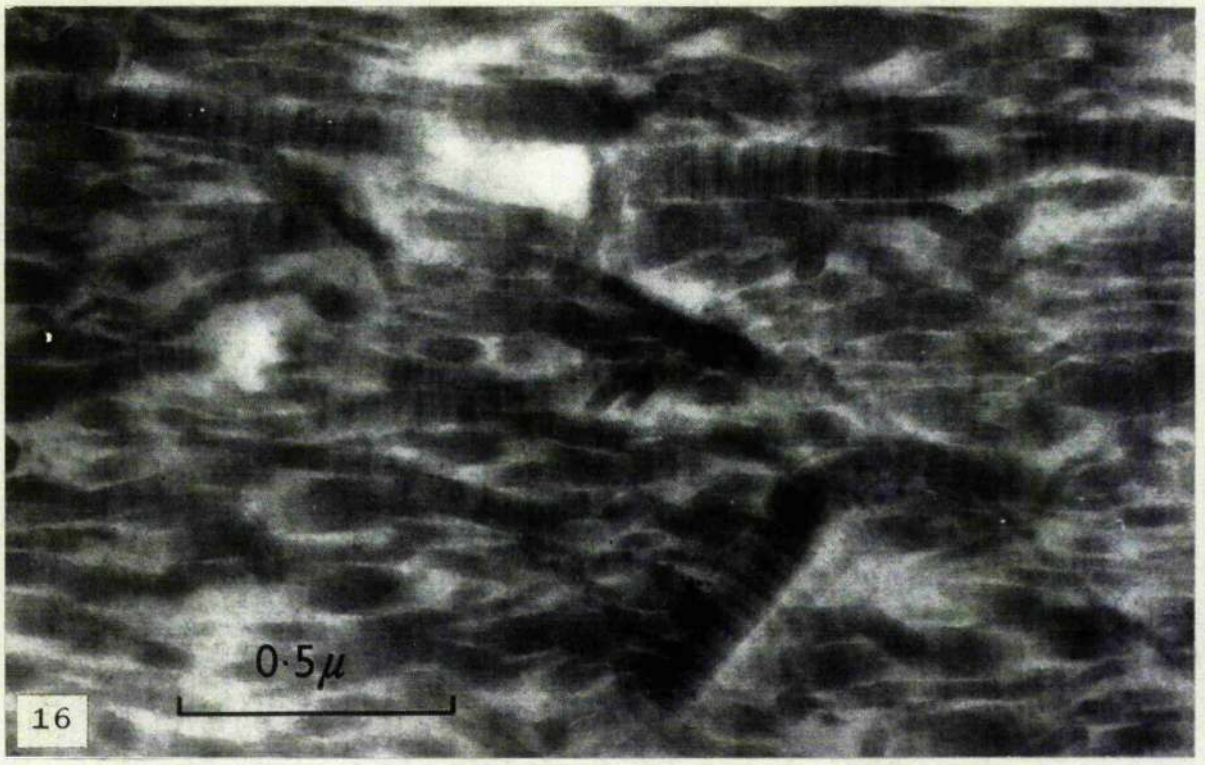
Figure 15



Articular cartilage of rabbit. Osmium fixation, stained uranyl acetate.

(x 13,000)

Figure 16

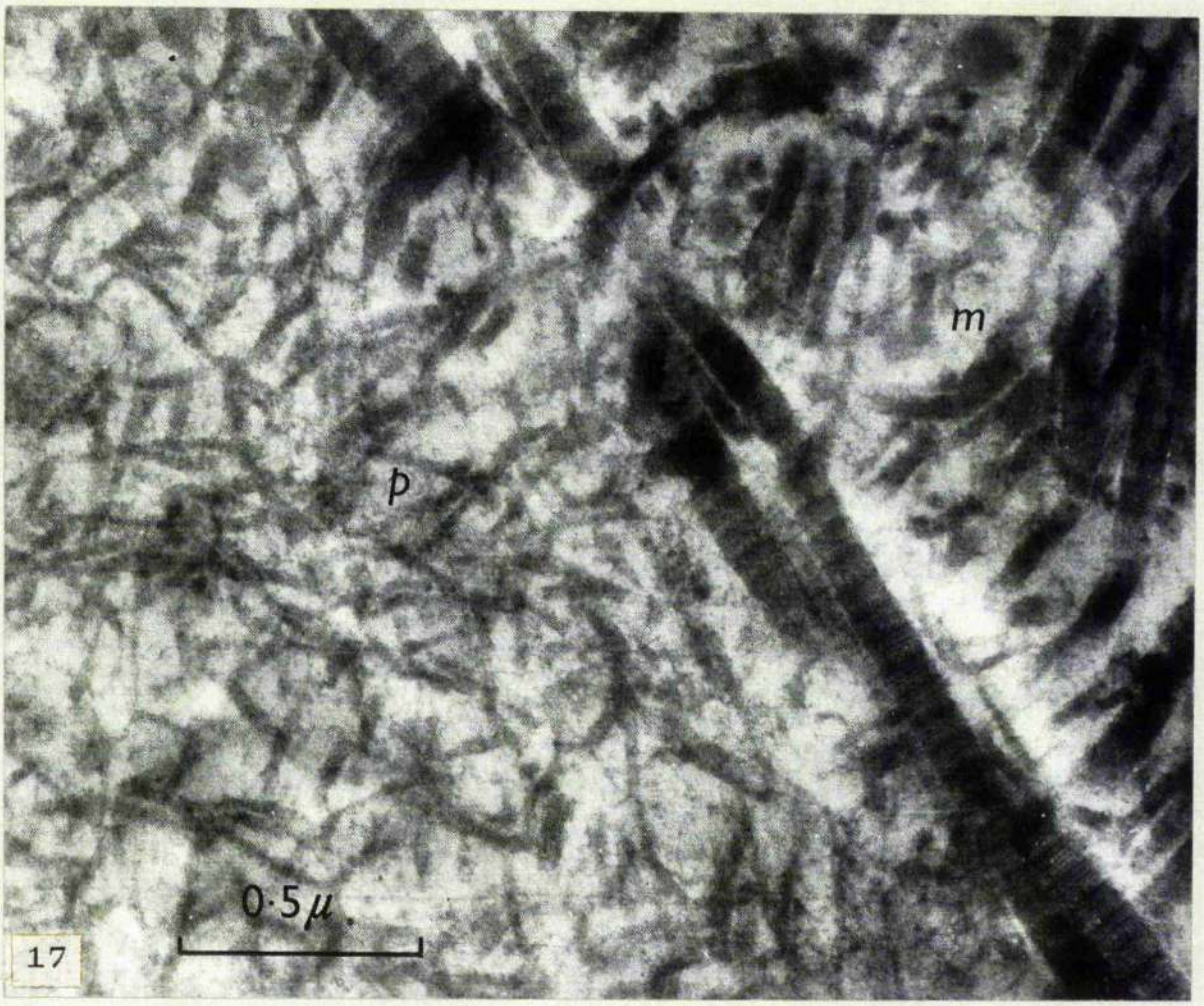


Bovine articular cartilage. Main matrix.

Osmium fixation, stained PTA.

(x 56,000)

Figure 17



Bovine articular cartilage. Osmium fixation, stained PTA.

(x 65,000)

structure is discussed in a later section of this paper. Between these 100- to 250-Å fibres, fine and rather tenuous fibrils are often evident so that the interfibrillar spaces of the pericellular zone are generally darker than those of the main matrix.

It seems clear that the dimensions of the pericellular zone are not artefactual, for it is quite distinct from the empty pericellular region which often surrounds a poorly fixed and contracted cell. The zone is not evident in the cartilage of small experimental animals (Figure 15) in which there tends, instead, to be a gradual increase in the average fibre diameter for some distance from the cell membrane (Silberberg et al., 1961; Revel and Hay, 1963). What is so characteristic of bovine articular cartilage is the abrupt increase of average fibre diameter from the pericellular zone to the main matrix.

Ultrastructure after bismuth staining

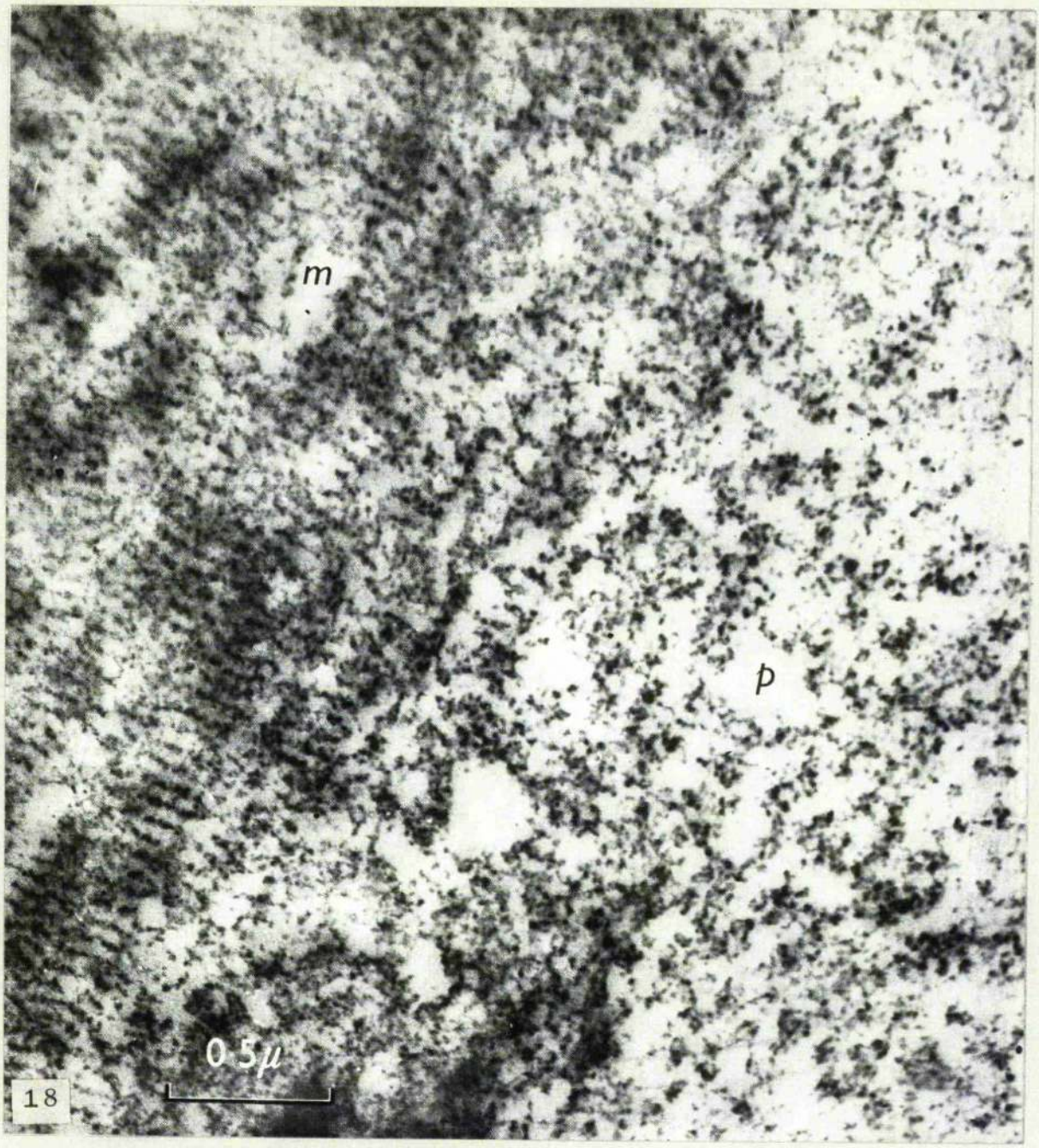
When unfixed articular cartilage is washed in 0.1M nitric acid and subsequently treated with 0.5% bismuth nitrate in 0.1M nitric acid at pH 1.2, the

cells of the tissue disintegrate so that only the nuclei and the plasma membranes remain recognizable. On the other hand, the matrix survives as a whole, and as is shown in Figure 18, the main matrix (m) and the pericellular zone (p) remain distinct in appearance and sharply demarcated from one another.

The main matrix (Figures 18 - 20) is largely occupied by well-defined rows of parallel dark bands. The bands are about 200 Å wide and are centred 550 Å apart. At high magnification (Figure 19) they are seen to be composed of minute particles which are about 30 Å in diameter.

Jakus (1961) has pointed out that in contrast to the 640 Å period of collagen dried on the grid, collagen in embedded and sectioned tissues tends to exhibit a shorter period of about 550 Å. Taking this difference into consideration, comparison of the dark particulate band in Figures 18 to 20 with the bands of particles previously noted in bismuth-stained PP-H strongly suggests that they too represent protein-polysaccharide macromolecules wound round the unstained collagen fibres of the tissue over the a and b_1 bands of each collagen period. Certainly the main matrix

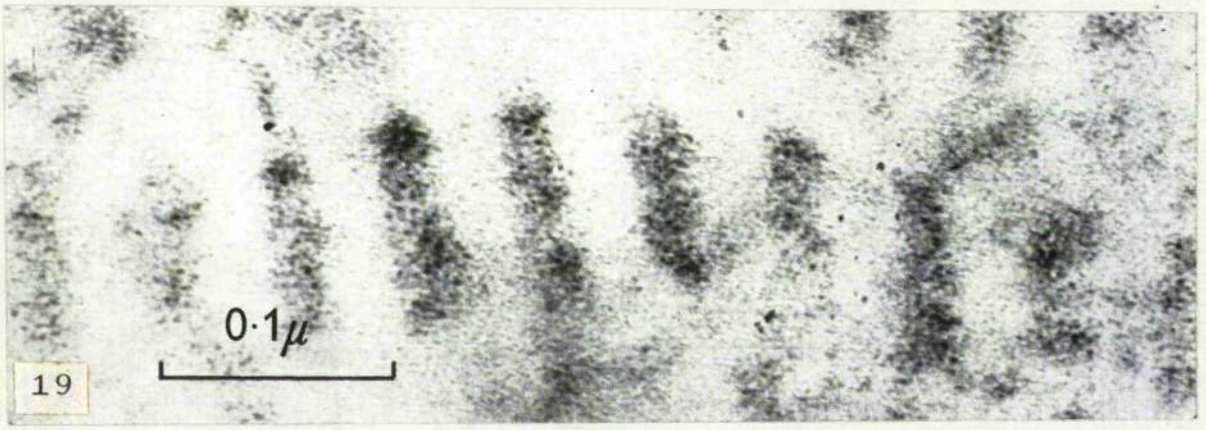
Figure 18



Bovine articular cartilage. Bismuth staining.

(x 46,000)

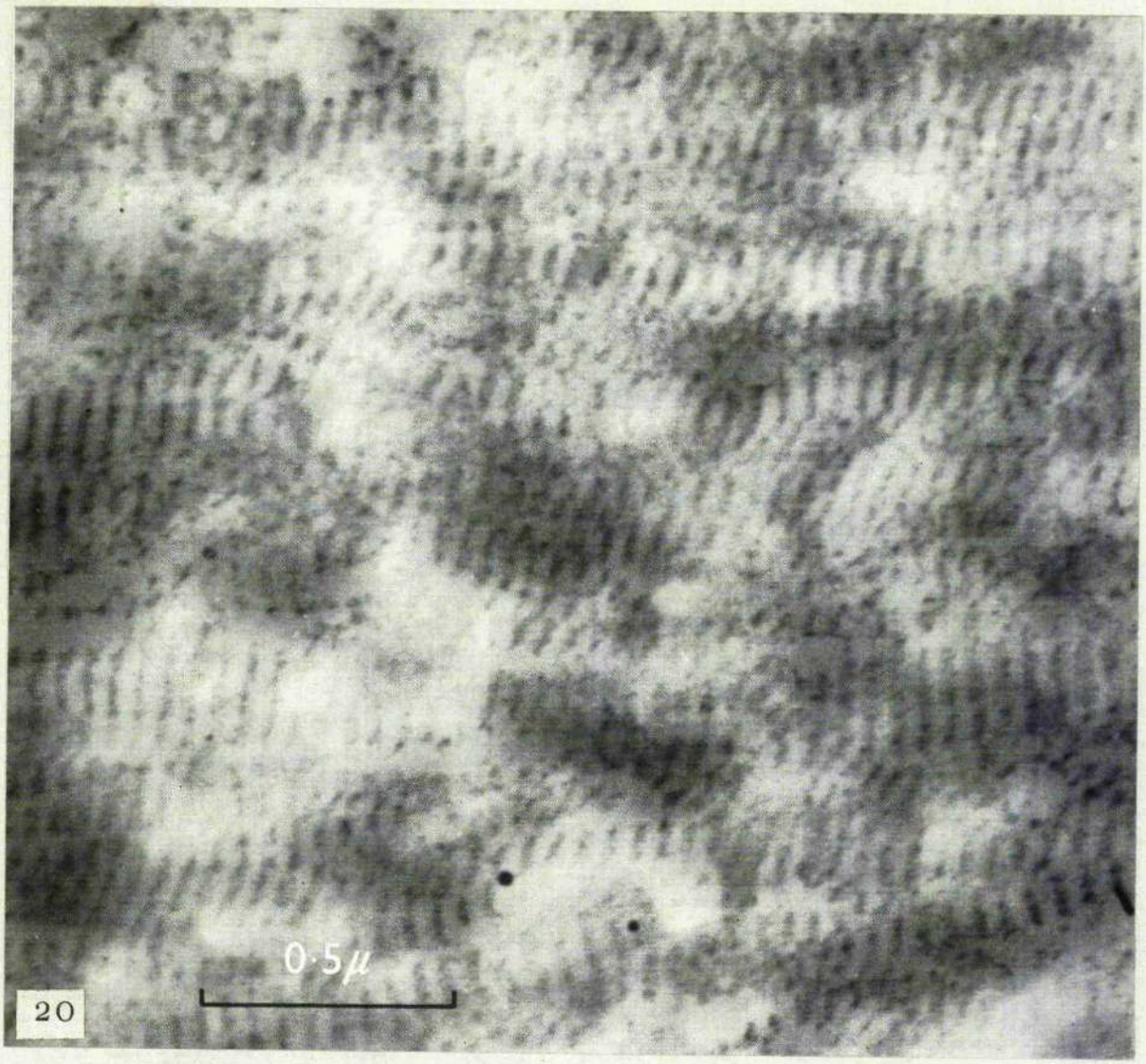
Figure 19



Bovine articular cartilage. Collagen fibre with attached protein-polysaccharide bands from main matrix. Bismuth staining.

(x 266,000)

Figure 20



Bovine articular cartilage. Central part of main matrix. Bismuth staining.

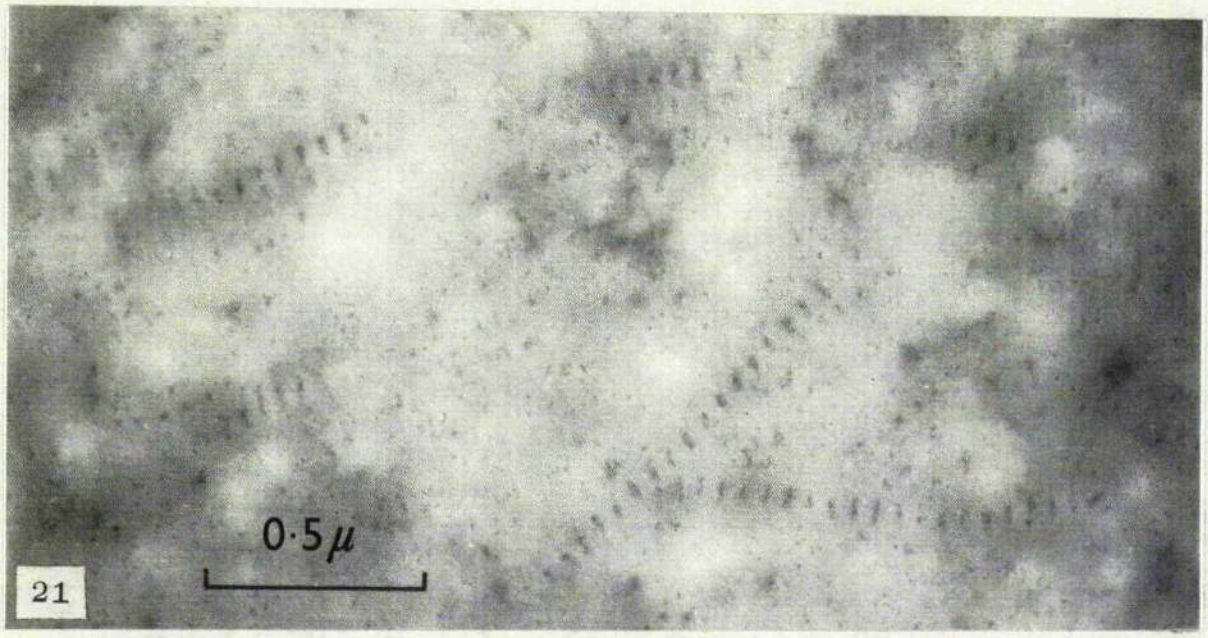
(x 62,000)

is characterized by single broad bands of particles rather than the twin bands exhibited by PP-H, but the overall width of 200 Å is similar. Moreover, it seems probable that the extent to which the particles are restricted to two separate rows depends on the degree to which the coiled CSA chains are approximated to the linear protein cores of the protein-polysaccharide macromolecules, and it appears reasonable to expect a closer approximation in the sprayed and dried material used in the examination of PP-H than in embedded and sectioned tissue.

The polysaccharide nature of the dark bands is confirmed by the effect of incubation of articular cartilage with testicular hyaluronidase before bismuth staining. After incubation for 24 hours at 37°C and pH 5, no staining of the tissue is evident, whereas after incubation for shorter periods isolated rows of dark bands persist in fields which are largely unstained (Figure 21).

It is a corollary to this concept that the protein-polysaccharide bands outline the unstained and invisible collagen fibres to which they are attached, and consequently that the lengths of the bands give a

Figure 21



Bovine articular cartilage. Main matrix.
Incubated with hyaluronidase for 12 hours.
Bismuth staining.
(x 50,000)

measure of the diameters of the collagen fibres. Assessed in this way, the collagen fibre diameter varies from 400 to 1500 Å, and it is apparent, therefore, that mainly as a result of swelling in an acid medium, the fibres are 70% wider in these bismuth preparations than they are in fixed cartilage.

In the pericellular zone (Figure 18), minute dark particles are arranged irregularly, in small groups and short linear rows, whereas the regularly spaced particulate bands which characterize the main matrix are not evident. This arrangement could represent a random distribution of isolated particles. Nevertheless, it is also in conformity with the arrangement to be expected in a section through randomly disposed protein-polysaccharide macromolecules. The latter concept is supported, first, by the fact that the pericellular particles are not apparent after treatment of the tissue with hyaluronidase, and secondly, by the demonstration by Gross, Mathews and Dorfman (1960) that the protein-polysaccharide of cartilage is metabolized as a single unit.

Particles exhibiting the arrangement described

above are not confined to the pericellular zone. They also extend into the main matrix where their number decreases progressively as the distance from a chondrocyte increases: many are evident at the cellular margin of the main matrix (Figure 18), whereas few are visible in its central part (Figure 20).

Physicochemical characterisation of the polysaccharides used in the in vitro collagen-binding experiments

Heparin

The molecular weight of heparin was calculated from data obtained in the ultracentrifuge, using an approach to equilibrium method. The ratio of the sedimentation and diffusion coefficients (S/D) x 10^7 was 2.1. The determination of $\bar{v}_{20}$ gave an average value of 0.42 ml./g. The refractive increment was 1.33 ml./g.

The molecular weight obtained from this experiment was 8,800. This value was confirmed by viscosity experiments giving a limiting viscosity number of 14.54 ml./g. The relation $[\eta] = 1.58 \times 10^{-3} M$ was used (Laurent, 1961).

The sulphur content was 16.05% of the ash- and moisture-free material.

Chondroitin sulphate

The chondroitin sulphate preparation gave a limiting viscosity number of 113.4 ml./g., corresponding to an average molecular weight of 44,000 when the relation $[\eta] = 2.58 \times 10^{-3} M$ was used (Mathews and Dorfman, 1953).

The sulphur content was 6.5% of the ash- and moisture-free material.

Dextran sulphate

The results of osmotic pressure measurements are shown in Figure 22 illustrating plots of π/C versus concentration of dextran sulphate. The straight lines were obtained by a least-squares method. The two curves represent data obtained with the same dextran sulphate preparation in buffers of different salt concentrations. The slopes of the curves decrease with increasing salt concentration as expected from the Donnan theory of membrane equilibrium.

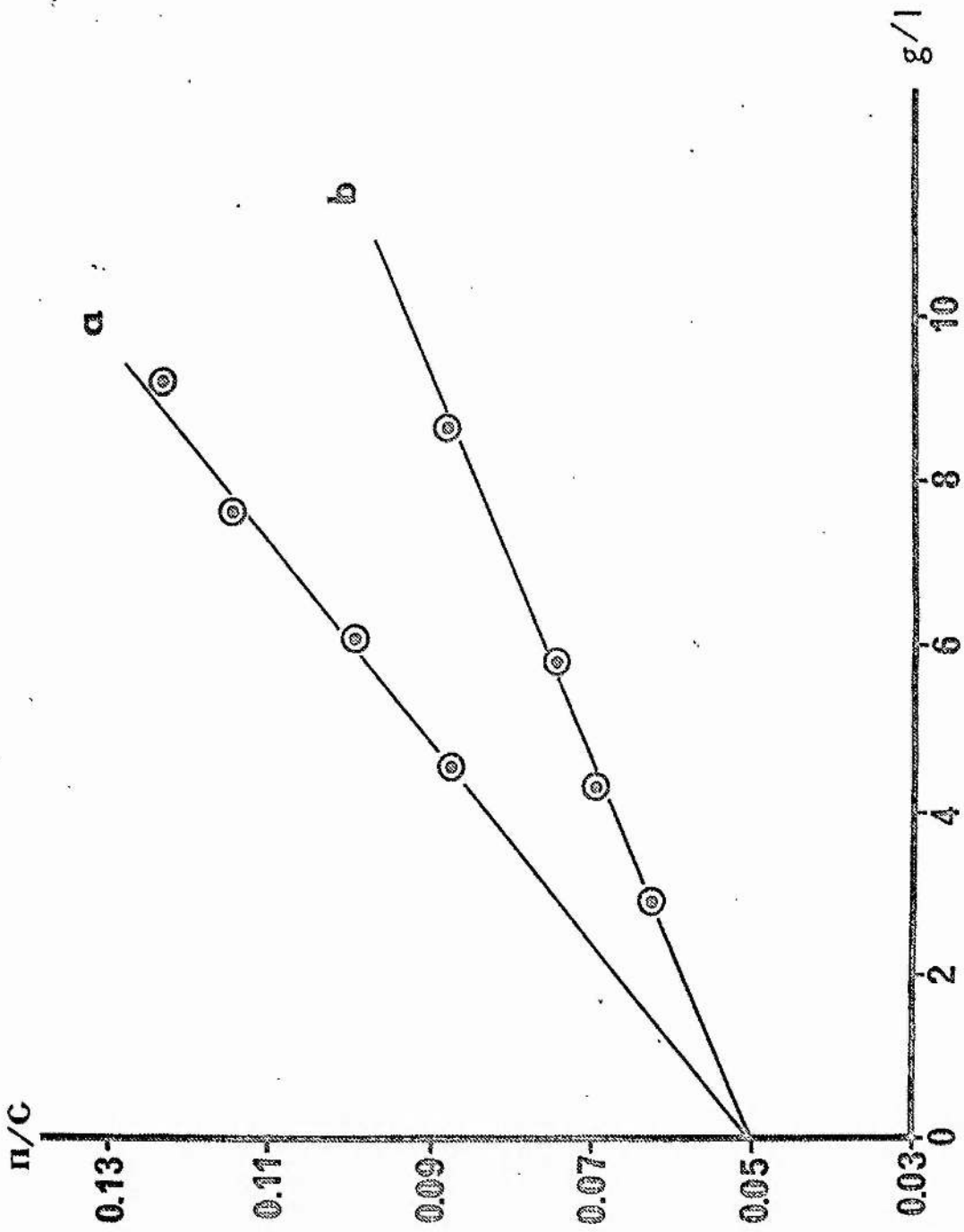
The number average molecular weight was calculated from the relation $M_N = RT/(\pi/C)_{C \to 0}$ where C is in grams/litre.

A number average molecular weight of 477,000 was obtained. The sulphur content was 18.3% of the ash- and moisture-free material.

Binding of polysaccharides by collagen in vitro

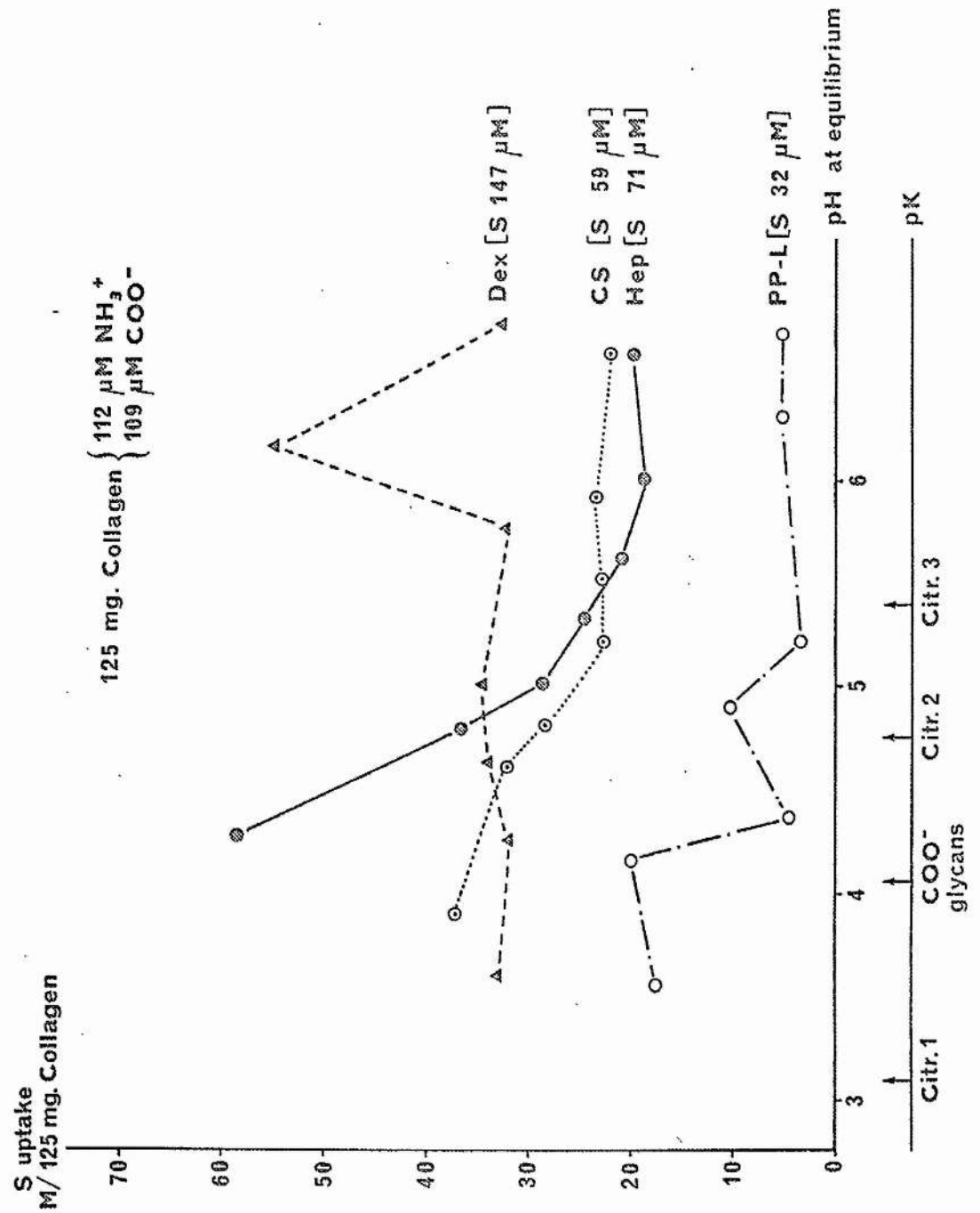
The diagram in Figure 23 shows the uptake of the various polysaccharides by collagen at different pH values. The uptake is expressed as μM of sulphur per 125 mg. of collagen. The pH recorded on the x-axis is that determined at equilibrium.

Figure 23



Reduced osmotic pressure of dextran sulphate in buffer solutions versus concentration.
Curve a) 0.1M phosphate buffer pH 6.5
Curve b) 0.1M phosphate buffer pH 6.5 + 0.4M NaCl.

Figure 23



Binding of polysaccharides by collagen in vitro

The chondroitin sulphate curve is similar to that reported by Einbinder and Schubert (1951) at low pH, but differs at high pH, where a significant uptake up to pH 7 can be observed.

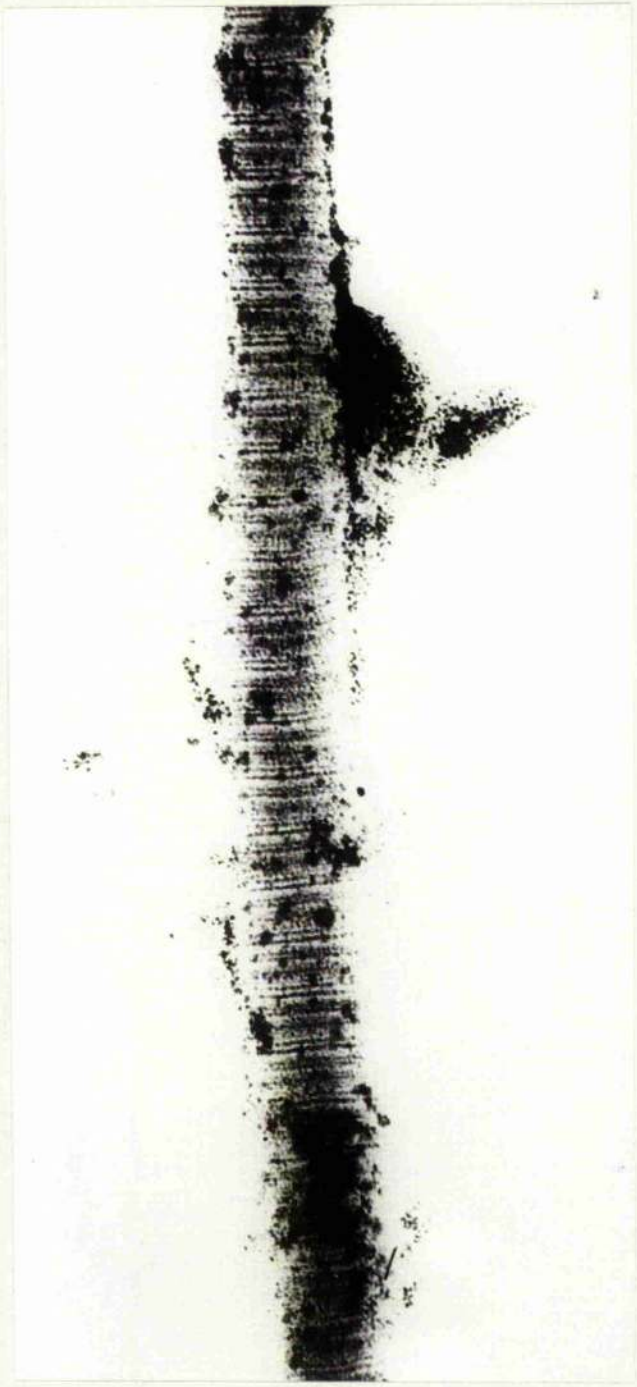
Heparin displays a similar pattern, but the two high-molecular-weight compounds behave rather differently. They show proportionally lower uptake at low pH but, particularly in the case of dextran sulphate, a high uptake at high pH is found with pronounced peaking effect.

Electron microscopy of the polysaccharide-collagen complexes

Figure 24 shows a collagen fibre with its bound heparin obtained at pH 5.0. The distribution of heparin molecules along the fibre has been analysed by preparing a densitogram which has then been compared with densitograms obtained from tendon collagen fibres stained with PTA at pH 2 or with uranyl acetate at pH 7. (Figure 25).

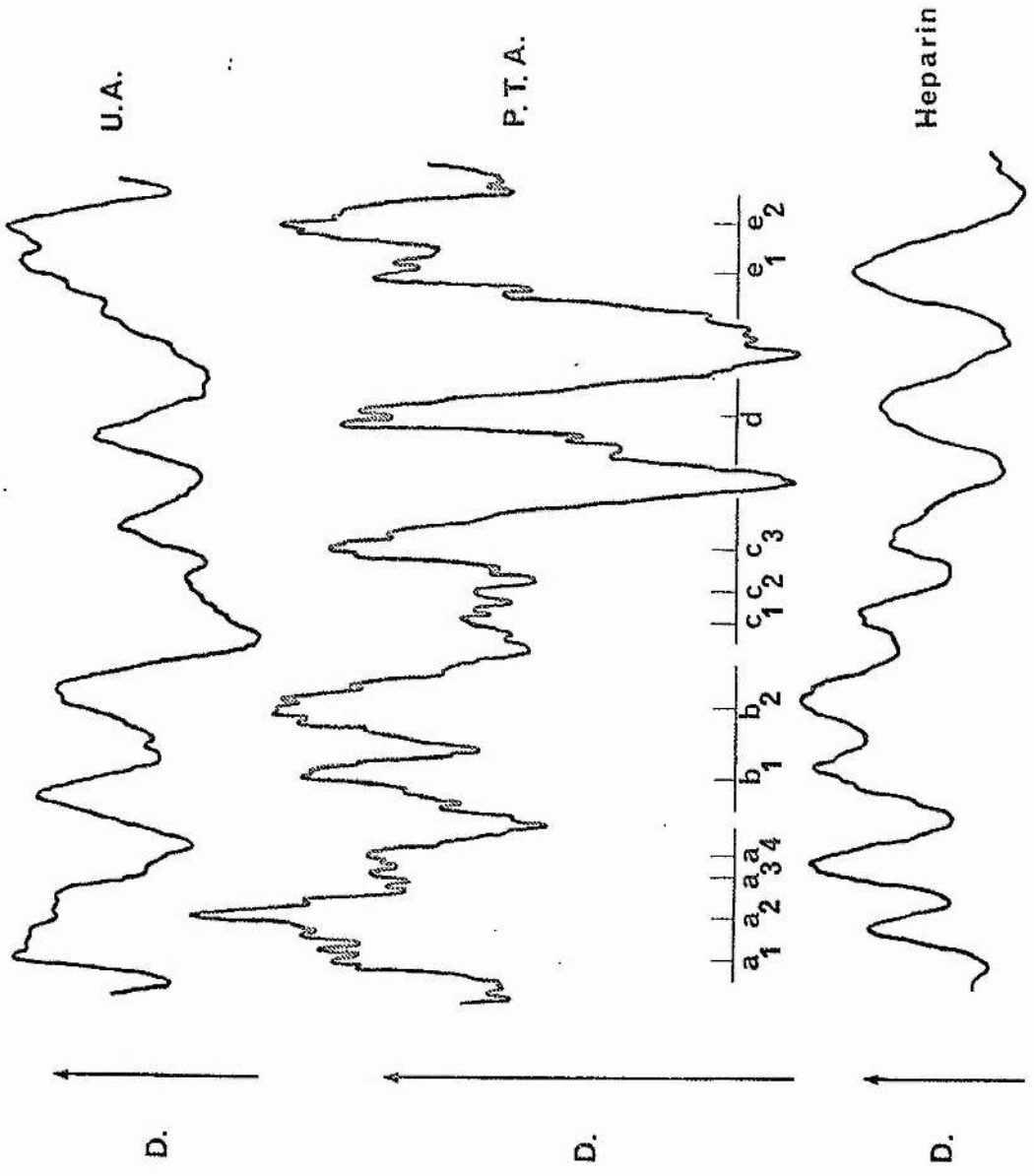
Figures 26, 27, and 28 show the appearance of polysaccharide-collagen complexes involving heparin at pH 6.6, dextran sulphate at pH 6.1, and PP-L-Bi at pH 4.3, respectively.

Figure 24



Heparin-collagen complex, pH 5. Bismuth staining
(x 70,000)

Figure 25

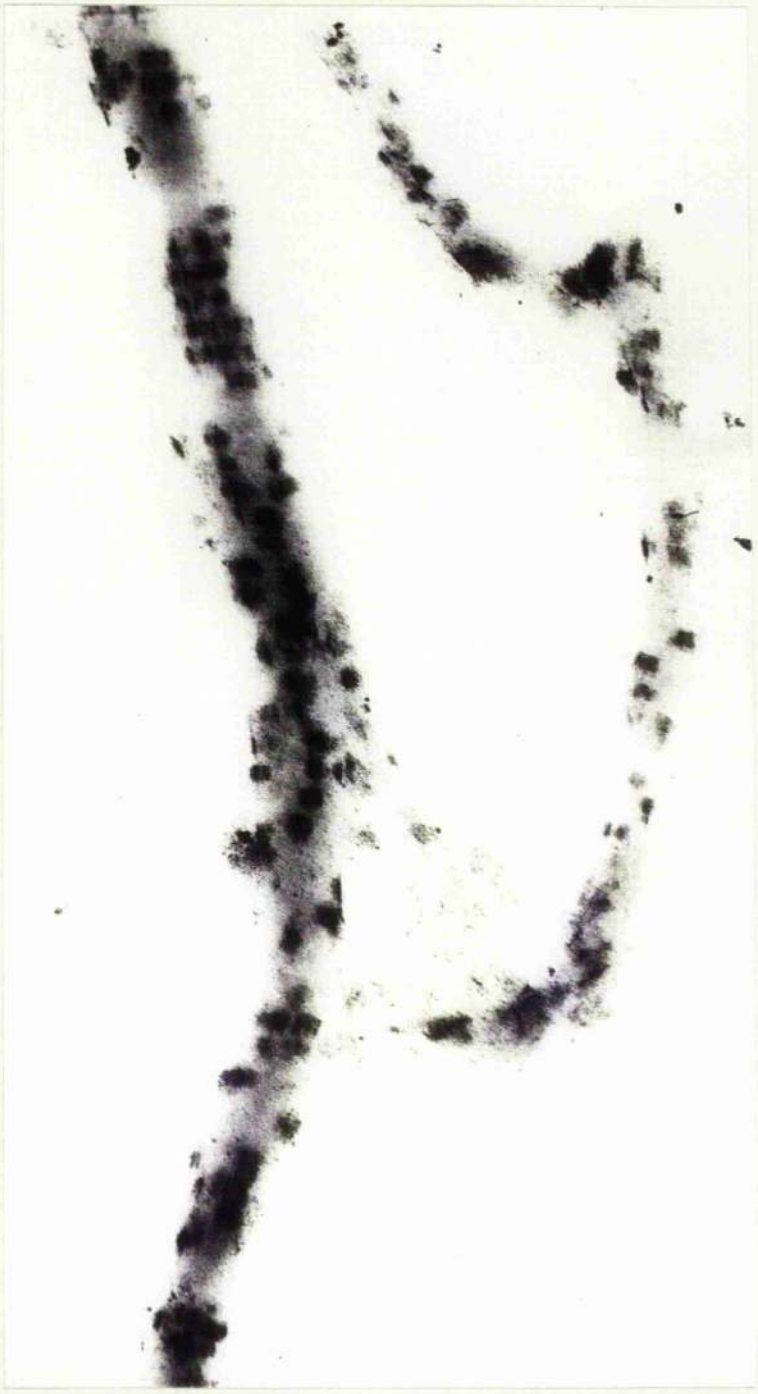


Densitogram of collagen fibre in figure 24 compared with densitograms obtained from collagen fibres stained with uranyl acetate and PTA.

Figure 26

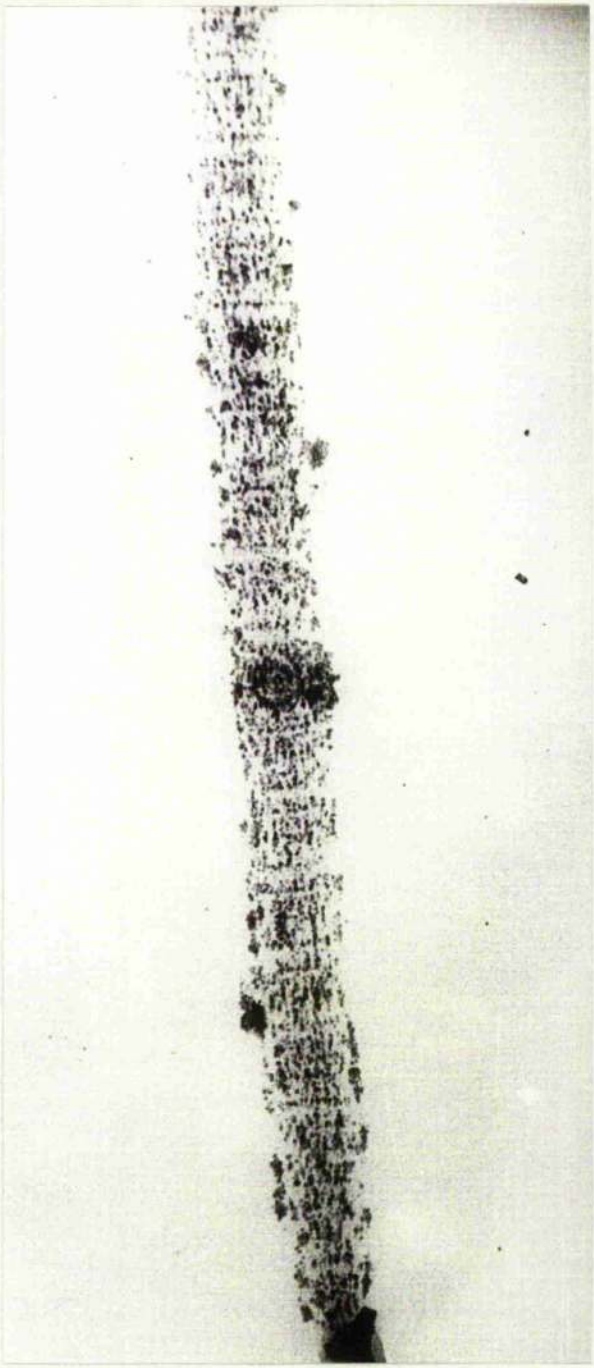
Heparin-collagen complex, pH 6.6. Bismuth staining.
(x 78,000)

Figure 27



Dextran sulphate-collagen complex, pH 6.1.
Bismuth staining (x 55,000)

Figure 28



PP-L-Bi-collagen complex, pH 4.3. Bismuth staining (x 150,000)

DISCUSSION

The investigation indicates that PP-L is the protein-polysaccharide complex of nasal cartilage, whereas PP-H consists in part of a mixture and in part of a combination of protein-polysaccharide and collagen.

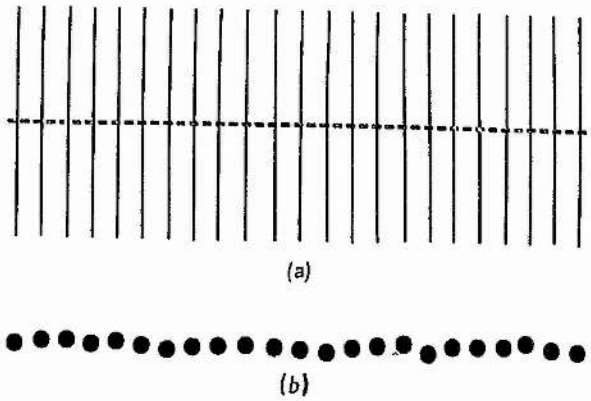
Throughout the investigation it has been noted that the appearance of the protein-polysaccharide macromolecules, and in particular their individuality, varies considerably in different circumstances. It is believed that these variations in morphology are to be related to variations in the degree of intermolecular cross-linking by bismuth ions, and that this, in turn, is dependent on the dielectric constant of the medium and on the extent of attachment of the protein-polysaccharide macromolecules to a rigid substrate. In the individual protein-polysaccharide macromolecules seen after precipitation by bismuth nitrate in acetone (Figure 4) the dark particles are considered to represent separate carbohydrate chains in coiled configuration, while the intervals between the particles represent the unstained protein core.

A schematic representation of the protein-polysaccharide macromolecule is given in Figure 29.

This electron microscopic appearance of the complex seems to be in keeping with the model proposed by Mathews and Lozaityte (1958), especially with respect to the distance between adjacent chondroitin sulphate chains; but there is disagreement about the length of the macromolecule. However, one of the preparations reported by Mathews and Lozaityte (1958) had a molecular weight, from light-scattering determinations, of 1.7×10^6 and a mean length of 2900 Å. This length was calculated from the root-mean-square radius of gyration. In a polydisperse system of rods, the molecular species to which this size refers are those larger than the z-average (Ehrlich and Doty, 1954). Moreover, some contribution to length could be due to the polysaccharide. It is likely therefore that the number-average length would be closer to the range 1100-1500 Å indicated by the present study.

Fitton Jackson et al. (1963) investigated the light fraction of an aqueous extract of chicken epiphysial cartilage having a sedimentation constant of 19.9. They demonstrated, by negative staining with PTA, that the material consisted of a dense mass of particles with

Figure 39



(a) Representation of the protein-polysaccharide macromolecule: the interrupted line indicates the protein core, and the solid lines CSA chains.

(b) Diagrammatic representation of the protein-polysaccharide macromolecule after bismuth staining. Dark particles are CSA chains in coiled configuration. The protein core is unstained.

dimensions of 45 x 55 Å, and in their view, these particles represented only the protein moiety of the protein-polysaccharide. It was noted that in many situations, rings of five or six particles with a total diameter of 165 Å were evident. The authors suggested that the protein core of the protein-polysaccharide macromolecule consists of a succession of such rings, piled one on top of the other, and that the polysaccharide chains are distributed around its peripheral surface.

Because in the present investigation only the carbohydrate moieties of protein-polysaccharide have been visualized, little significance can be given to the similar size of the particles in Figures 3 and 4 and those demonstrated by Fitton Jackson *et al.* (1963). But it is apparent that the morphology of the protein-polysaccharide macromolecule which is suggested by Figures 4, 5 and 6 is quite out of keeping with the concept of a protein core 165 Å in diameter.

The electron microscopic examination of the heavy fraction of the aqueous cartilage extract - that is, the PP-H fraction of Gerber *et al.* (1960)

shows that protein-polysaccharide macromolecules of similar morphology exhibit a transverse surface attachment to collagen fibres over two closely adjacent sites in each 640 Å period. In the same material, counterstained with PTA, the sites of attachment conformed to the a and b_1 bands in the notation of Schmitt and Gross (1948)(Figure 30).

It has been suggested by Hodge and Petruska (1963) that in native collagen fibres, the length of each tropocollagen molecule is 4.4 times that of the native collagen period, and that holes consequently exist between the ends of adjacent molecules in the region between the c_2 and a_2 bands. If such holes do in fact exist, it appears that the protein-polysaccharide on the a and b_1 bands are not directly related to them.

Fitton Jackson and Randall (1956a,b) have demonstrated that in embryonic bone the earliest mineral crystals are precisely located between the d and the ab bands of each native collagen period and therefore within the region of the intermolecular holes discussed above. The present investigation indicates that the position of these initial mineral

Figure 30

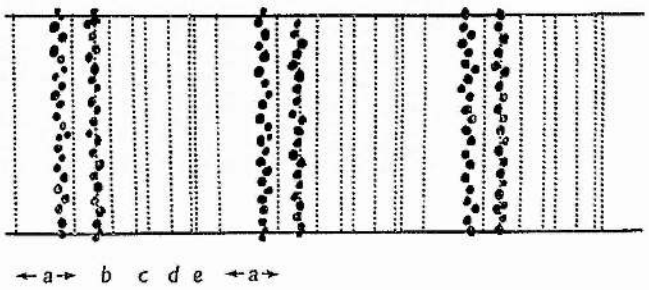


Diagram of the attachment of protein-polysaccharide to a collagen fibre in PP-II. Solid lines indicate the margins of the fibre. Interrupted lines indicate the PTA band pattern. The dark particles represent the CSA chains of protein-polysaccharide macromolecules which are attached over the a and b_1 bands of each collagen period.

crystals lies between adjacent pairs of protein-polysaccharide locations and this leaves open the interpretation of the part played by protein-polysaccharide in the early stages of the calcification process (Sobel and Burger, 1954; Sobel, 1955; Glimcher, 1959; Hamerman and Schubert, 1962).

The protein content of PP-L decreases from an average value of 18.6% to 14.1% on purification of the protein-polysaccharide complex by precipitation with bismuth nitrate in acetone. The alternative purification by precipitation with cetylpyridinium chloride and selective resolubilization of the complex produces a material of intermediate protein content (16.0%). However, as shown in Table 2, the composition of the protein moiety changes very little on purification, the major features being a lower arginine content and the absence of tryptophan, methionine and cystine in both purified protein-polysaccharide complexes. Some of the slight differences in amino acid composition that are seen in different samples and preparations can be attributed to the contamination of PP-L-C by free amino acids and peptides that are removed by acetone at low pH,

and by trace amounts of at least two proteolytic enzymes. Steven and Tristram (1962) pointed out that acetone extraction releases free amino acids and peptides from highly purified collagen. The presence of these compounds might therefore be considered as a physiological feature of connective tissues.

Even taking into account the difficulties of making deductions from overall amino acid analyses, the basic similarity in amino acid composition of crude and purified preparations, in spite of the decrease in protein content with purification, suggests that a proportion of the protein-polysaccharide macromolecules in PP-L-C may have a decreased chondroitin sulphate content. The latter could be naturally occurring degradation products of metabolic turnover in which some of the chondroitin sulphate chains have been cleaved from the protein core and then excluded from the domain of the macromolecule.

Examination of the amino acid composition of PP-L-Bi, expressed as residues/1000 residues (Table 2), reveals the existence of several groups in which the amino acids are present in approximately equimolar concentration. Serine, proline and glycine each

appear to occur with the frequency of 1 in 10 residues; aspartic acid and leucine as 1 in 12 residues; threonine, alanine and valine as 1 in 16 residues and, finally, phenylalanine, lysine and arginine as 1 in 29 residues. Equimolarity of serine, glutamic acid, proline and glycine was reported by Anderson, Hoffman and Meyer (1965) in chondroitin 4-sulphate preparations obtained by proteolytic digestion of chondromucoprotein from nasal cartilage and characterized by an amino acid content of approximately 3%. A similar pattern was found by the same authors in samples of chondroitin 6-sulphate obtained from shark cartilage. But when DNP derivatives of the latter preparation were examined, several amino acids appeared to be N-terminal, indicating inhomogeneity of the small peptide chains.

These data suggest that the protein core of PP-L may be made up from a limited number of peptide 'sub-units' of slightly differing amino acid composition and sequence, but with the same amino acids involved in the linkage region with the polysaccharide.

The amino end-group analysis carried out on PP-L-Bi (Table 3) shows that six amino acids can

be consistently detected as α-DNP derivatives. The quantitative determination of their concentrations reveals that they are present as approximately integral molar multiples of glycine. These findings alone might suggest that PP-L is a mixture of protein-polysaccharide macromolecules differing in their protein backbones. But this hypothesis cannot explain the striking similarity in amino acid composition of chondromucoprotein preparations purified by different methods. An alternative interpretation could be that the protein core of PP-L is made up of peptide 'sub-units', the sequential arrangement of which in the macromolecule is not strictly determined. If this were so, each 'sub-unit' would occupy the external position in the macromolecule at the free amino end with a frequency proportional to its occurrence in the whole protein core.

If this second hypothesis is correct, the number-average molecular weight of the protein-polysaccharide macromolecule can be calculated from the total number of moles of N-terminal amino acids/10^6g. of PP-L. The value of 6.3×10^5, so obtained, is in good agreement with that (5.5×10^5) reported by Buddecke *et al.* (1963) in their study on PP-L purified with

cetylpyridinium chloride. The similarity between between PP-L-Bi and PP-L-CPC is emphasized in the present work by the identical values for their $S^{o}_{20.w}$.

Since the protein content of PP-L-Bi is 14.1%, the molecular weight of the protein core can be calculated as 8.9×10^4, leaving a total molecular weight of 5.4×10^5 for the carbohydrate moiety. The chain weight of chondroitin 4-sulphate, isolated by papain digestion, has been reported as ranging between 2.2×10^4 and 2.8×10^4 (Mathews, 1956; Partridge et al., 1961; Buddecke et al., 1963). Therefore some 20-24 chondroitin 4-sulphate chains should be present in the protein-polysaccharide complex. These findings are in remarkable agreement with the results of the electron-microscopic examination of PP-L-Bi, already discussed, where 20-25 particles, interpreted as chondroitin sulphate chains, were visualized to be arranged in single rows, 1100-1500 $\mathring{A}$ long.

Although the bismuth method of staining the protein-polysaccharide of cartilage for electron microscopy appears to be the only procedure available at the present time, it has to be recognised that it

does have one considerable disadvantage. Preliminary fixation by osmium tetroxide, glutaraldehyde or formalin appears to block entirely the attachment of bismuth to protein-polysaccharide. Thus, because of the necessary absence of fixation, the method does not permit any study of the chondrocytes or of the intracellular location of protein-polysaccharide during its synthesis. And although the intercellular matrix as a whole survives the lack of preliminary fixation, the diameters of its collagen fibres are enlarged by some 70%. The postulated mechanism of the method has been discussed previously. In particular, it has been suggested that the CSA chains of the protein-polysaccharide macromolecules are made evident only because of the neutralisation of their net charge by bismuth, and their consequent adoption of a coiled configuration. But although this coiled configuration permits their visualisation, it is not, of course, representative of the condition of the CSA chains *in vivo*. On the contrary, it seems probable that in untreated cartilage the CSA chains extend into the interfibrillar spaces and there bind to themselves a large volume of water. From the effects of trivalent cations on the elastic properties of articular cartilage

which have been noted by Sokoloff (1963) it is probable that this domain of bound water is liberated when bismuth is bound to CSA.

The main matrix of articular cartilage contains mature native collagen fibres which vary evenly in diameter from 250 to 900 Å throughout the region. On the other hand, the nature of the small fibres which are evident in the pericellular region after osmium fixation is difficult to interpret. Although it seems very probable from their form that they are collagenous, they have not been noted to exhibit the characteristic staining pattern which would be expected in collagen fibres of similar size in other tissues. It may be that in vivo the zone is largely occupied by monomeric collagen units rather than aggregated fibres, and that the fibres evident in section are the result of an irregular polymerization occurring during fixation and embedding. This hypothesis is supported by the broad similarity of the pericellular fibres to the fibrous inclusions which have often been noted in the Golgi vacuoles of chondrocytes (Godman and Porter, 1960; Revel and Hay, 1963).

After bismuth staining, protein-polysaccharide is evident in articular cartilage matrix both as free macromolecules and as macromolecules which are attached to collagen fibres in a regular periodic manner. In the pericellular zone all the protein-polysaccharide is free. On the other hand, in the main matrix a high proportion of the protein-polysaccharide is periodically bound to collagen and the free protein-polysaccharide diminishes in amount as the distance from a cell increases. This distribution of protein-polysaccharide is in conformity with existing information concerning the passage of this substance from the chondrocytes through the extracellular matrix (Godman and Lane, 1963; Fewer, Threadgold and Sheldon, 1964) and with its turnover in the tissue (Fitton-Jackson, 1964). The gradient of free protein-polysaccharide concentration in the main matrix suggests that these macromolecules are in the process of migration from the pericellular pool to evenly distributed turnover sites throughout the main matrix.

The appearance of the main matrix of articular cartilage differs markedly from that of nasal cartilage.

In the latter tissue there are comparatively few collagen fibres and although each of these appears to carry its full complement of protein-polysaccharide, the amount of free protein-polysaccharide is comparatively very large. This morphological distinction between the two tissues is, of course, reflected by their chemical composition (Table 4). It seems probable that the greater proportion of protein-polysaccharide, and therefore of water, attached to the rigid substrate of collagen in articular cartilage, is to be correlated with the more intense stress environment of this tissue.

The examination of the titration curve of collagen (Bowes and Kenten, 1948) shows that, at low ionic strength, the net charge is practically zero above pH 4 so that one might be led to assume that no electrostatic binding could take place, at pH values near to the physiological one, between collagen and protein-polysaccharide complex in cartilage. But this ignores that the uneven charge distribution along the fibre axis, typical of collagen, is such that alternating regions of positive and negative overall charge could exist even at pH near the isoelectric point. To prove this point, samples of collagen have been stained

at pH 2 with PTA, which specifically reacts with guanidino groups, while others were stained at pH 7 with uranyl acetate, which reacts with carboxylate ions. Densitograms have then been prepared from the electron micrographs of stained fibres isolated from both preparations. The top diagram in Figure 31 shows the position and charge density of the peaks corresponding to the positive charges in the 640 Å period. The height of the lines is proportional to the area of each individual peak in the densitogram, expressed as a percentage of the total area over a 640 Å period. The lower diagram shows the position and charge density of the peaks corresponding to the negative charges.

The relative position of the diagrams is tentatively adjusted for the best fit of the greatest number of corresponding lines. It is evident that in the region corresponding to the $\underline{a}_3\underline{a}_4$ and $\underline{c}_1\underline{c}_2$ positive bands, positive charges will not be counter-balanced by negative charges under all conditions of ionization. It is suggested that a charge distribution of this kind might have a considerable bearing on the mechanism of binding of charged macro-molecules by collagen under physiological conditions.

Figure 31

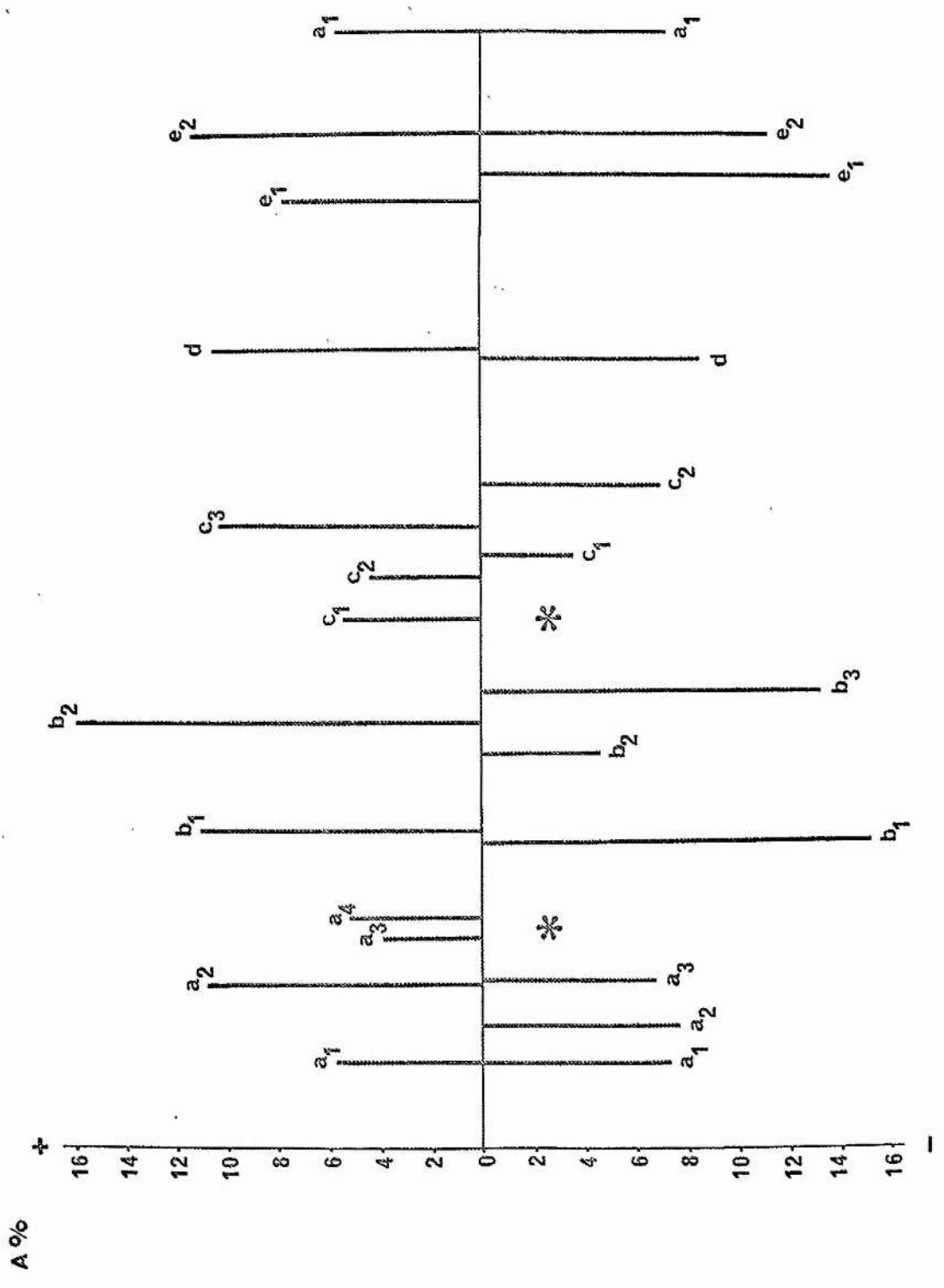


Diagram showing position and charge density of the intraperiod bands of collagen.

This hypothesis is supported by the observation that collagen is able to bind sulphated polysaccharides at pH values near to the isoelectric point. Differences in uptake displayed by different polysaccharides are probably due to factors related to their charge, size and structure.

The electron microscopic examination of the various polysaccharide-collagen complexes is also very interesting in this respect. It appears, as a general pattern, that at low pH there is a very high uptake with random orientation.

At intermediate pH (around pH 4-5) the behaviour depends very much on the size and shape of the macromolecules. Heparin, in fact, probably because of its small size, fully occupies all available sites, whereas PP-L tends to be already restricted to two narrow zones in each collagen period. It is quite interesting to consider that although the majority of the latter macromolecules are arranged longitudinally, some are instead orientated with their long axis at right angles to the collagen fibre axis, as <u>in vivo</u>.

At pH above 6, the binding of both heparin and dextran sulphate is restricted to a single zone in the 640 Å period. At this pH, as predicted by the analysis of the charge profile of collagen, there appears to exist in each period a narrow positive region surrounded by two broad negative zones. The presence of this inhomogeneous electrical field on collagen develops forces which cause an orientated movement of charged macromolecules towards the centres of inhomogeneity.

As pure speculation, it can be suggested that, at physiological pH, PP-L which shows high degree of asymmetry, on approaching the collagen fibre will be orientated by the electric charge distribution so that its long axis will be at right angles to the fibre in which position it will zip over the ring of positive charges, being prevented from attachment in the longitudinal orientation by the surrounding negative charges.

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